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THE UNIVERSITY OF ALBERTA

ISOLATION AND CHARACTERIZATION OF A PROLACTIN BINDING PROTEIN IN HUMAN MILK

by

(C)

DIANA ROLANDE MAGER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF FOODS AND NUTRITION

EDMONTON, ALBERTA

SPRING 1989

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ISBN 0-315-52817-6

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TITLE OF THESIS: Isolation and Characterization of a Prolactin Binding Protein in Human Milk

DEGREE: Master of Science

YEAR THIS DEGREE AWARDED: 1989

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ISOLATION AND CHARACTERIZATION OF A PROLACTIN BINDING PROTEIN IN HUMAN MILK submitted by DIANA ROLANDE MAGER in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in FOODS & NUTRITION (FACULTY OF HOME ECONOMICS).

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TO MY MOTHER FOR HER LOVE AND ENCOURAGEMENT

ABSTRACT

A prolactin binding protein was isolated and characterized in milk from mothers delivering prematurely (23-34 weeks) and at term. To assess whether a relationship exists between maternal hormonal status and the nutrient composition of milk, comparison of the prolactin receptor content of milk microsomal membranes with the nutrient composition of milk at different stages of gestation and duration of lactation was made. Binding properties of prolactin receptor were also compared with the fatty acid composition of microsomal membrane phospholipids to assess the role of changes in membrane composition in the functional properties of the receptor.

Treatment of milk microsomal membrane with 5M $MgCl_2$ to dissociate endogenously bound prolactin resulted in a significant increase in specific binding. Specific binding of prolactin to its receptor changed with incubation time, temperature and concentration of microsomal membrane protein. Binding of prolactin was saturable, reversible and specific for human prolactin.

Total receptor content in milk increased with duration of lactation. No apparent effect of gestation on receptor content and binding affinity was observed over the course of the study. Prolactin receptor content was negatively correlated with the concentration of prolactin in milk over the length of lactation. A negative correlation between the concentration of prolactin and the lipid and lactose content of milk was observed over the course of this study. Prolactin receptor content, however, was weakly correlated with the protein and lactose content of milk. These results in-

dicate that a relationship exists between maternal hormonal status and the nutrient composition of milk over the duration of lactation..

Increasing levels of polyunsaturated fatty acids (w/w) in microsomal phospholipids were associated with an increase in the number of available prolactin binding sites. These results indicate that changes in the fatty acid content of membrane phospholipids may have a direct effect on prolactin action.

This thesis demonstrates that a relationship between maternal hormonal status and the nutrient composition of milk exist. Further research is required to study the effect of maternal diet on prolactin receptor function.

ACKNOWLEDGEMENTS

I wish to thank Dr. Tom Clandinin for his time, guidance and encouragement throughout the course of this study.

I extend my sincere appreciation to Dr. Jaya Venkatraman for her friendship and technical assistance. Thanks are extended to Christina Ko and Elizabeth Woo for their encouragement and friendship.

I greatly appreciate the invaluable advice and help provided by Dr. Goh in this study. Thanks are extended to Mrs. Hinman for her help in preparing this manuscript.

Financial support for this project provided by Natural Science and Engineering Research Council of Canada is gratefully acknowledged.

Finally, a very special thanks to my husband, Li-Wen Yeow, for his support and help in preparing the illustrations for this thesis.

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I. Factors Influencing Milk Composition: The Role of Prolactin

A. Introduction

Nutrient differences exist in milk produced by mothers delivering prematurely and at term (Atkinson et al., 1978; Lemons et al., 1982). These differences are thought to be influenced by maternal hormonal status (Cousins et al., 1977; Chappell & Clandinin, 1985) and maternal diet (Finley et al., 1983; Davila et al., 1983; Lammi-Keefe & Jensen, 1984). The primary hormone involved in the initiation of lactation is prolactin. The mechanism by which prolactin exerts these effects is not clear. Recent evidence indicates that an internalization of the prolactin receptor-complex at the basal plasma membrane may be responsible for initiation of these actions (Ymer et al., 1987; Nolin & Witorsch, 1976; Dunaif et al., 1982). It is possible that analysis of milk for prolactin and/or a prolactin binding protein may be useful in assessing prior hormonal events leading to lactation. Additionally, comparison of the concentrations of these factors in milk with the nutrient composition of milk at different stages of gestation and duration of lactation may help explain how change in maternal hormonal status alter lactogenesis.

B. Factors Influencing Milk Composition

Milk contains a large number of nutrients (Atkinson et al., 1978; Lemons et al., 1982), growth factors, immunoglobulins, enzymes and hormones (Strbak 1985; Gupta 1983; Grosvenor & Whitworth, 1983; Taketani & Mizuno, 1985). The composition of milk varies among individuals and is

affected by several factors. Diurnal variations in composition occur within-and-between feedings (Moran et al., 1983; Ferris & Jensen, 1984; Jensen et al., 1985). Changes in composition are also observed over the duration of lactation and at different stages of gestation (Atkinson et al., 1978 ;Lemons et al., 1982; Moran et al., 1983; Ferris & Jensen, 1984). Maternal hormonal status (Healy et al., 1980; Chappell & Clandinin, 1985; Kulski & Hartmann, 1983; Kulski et al., 1977) and dietary intake (Ferris & Jensen, 1984; Jensen et al., 1978) are thought to be responsible for these differences in composition. However, differences in milk composition reported in the literature may also be due to the method of milk collection, the handling and storage of milk specimens and the methods of analysis used to determine composition as these varied among investigators (Jensen et al., 1985; Lemons et al., 1982; Chappell & Clandinin, 1984).

Effect of Collection Procedures and Handling of Milk Specimens

Containers used to store milk may affect the accuracy of nutrient analysis. In particular the immunological components of milk may adhere to pyrex, polyethylene and polypropylene containers (Hamosh et al., 1984). The temperature at which milk should be stored from the time of collection until analysis depends upon the substance to be analyzed. For example, frozen storage does not significantly alter the levels of biotin, niacin, folic acid (Friend et al., 1983), total fatty acid content (Chappell et al., 1985), the relative percentage of each fatty acid and milk protein levels (Bjorksten et al., 1980). However, freezing and thawing and repetitive cycles of freezing and thawing of milk result in a greater hydrolysis of

triglycerides (Chappell et al., 1985) and a disruption of fat globules in the milk (Wardell et al., 1981). Storage of milk at -70°C in the presence and absence of a lipase inhibitor resulted in significant differences in fatty acid profiles and levels of free fatty acids (Chappell et al., 1985). In the absence of a lipase inhibitor, higher levels of free fatty acids in the milk were reported, suggesting that the freeze-thaw cycle stimulated lipoprotein lipase activity.

Heating of milk to temperatures above 37°C results in a progressive loss in lipase and esterase activity (Wardell et al., 1984), the elimininination of pathogens, damage to the antimicrobial properties in milk (Bjorksten et al., 1980) and losses in essential fatty acids by autooxidation (Wardell et al., 1984). Reports of a decrease in the percentage of linoleic and linolenic acid in milk triglycerides following heating of milk to 62.5°C have been attributed to the deactivation of milk lipases (Wardell et al., 1984).

Thus, whatever procedure is used to collect milk specimens, the methods employed must be rigidly standardized to account for compositional changes that occur naturally (Jensen et al., 1985). Frozen storage of samples in the presence of lipase inhibitors are indicated to prevent or minimize artifactual determinations of milk fatty acids.

Effect of Gestational Age and Duration of Lactation

Although controversy in the literature exists regarding the nutrient composition of milk at different stages of gestation and lactation, the following observations have been reported. The concentration of fat, protein,

nitrogen, energy and electrolytes in preterm milk is generally higher than that found in milk produced by mothers delivering at term (Atkinson et al., 1978; Lemons et al., 1982; Ferris & Jensen, 1984; Moran et al., 1983). Preterm milk also contains higher levels of medium chain triglycerides (Bitman et al., 1983). Variations in the nutrient composition of milk also occur most dramatically with the onset of lactation from immature (3-5 days) to mature milk (15-18 days) (Ferris & Jensen, 1984). For example, the fat content of milk increases from 2-3% w/w (colostrum) to 4-6% w/w (mature milk) within the first two weeks of lactation (Lemons et al., 1982; Moran et al., 1983). The concentrations of protein, vitamins A, D, E, and K, and the trace minerals also decrease over this period (Moran et al., 1983; Dewey et al., 1984). Longitudinal decreases in milk concentrations of zinc, copper, potassium, protein, iron and sodium continue for the first six months of lactation, while concentrations of lactose, fat, calcium and magnesium remain unchanged (Dewey et al., 1982).

The changes observed in the nutrient composition of milk at different stages of gestation and duration of lactation appear to reflect the energy requirements of the growing infant (Chappell et al., 1983; Ferris & Jensen, 1984). For example, the higher levels of medium chain triglycerides in preterm milk may enhance hydrolysis and absorption of milk fats in the immature gastrointestinal tract of the preterm infant (Ferris & Jensen, 1984). These differences may reflect the functional immaturity of the preterm mammary gland (Bitman et al., 1983). Chappell and coworkers (1985) found no difference in the free fatty acid content of preterm and fullterm milk, although an effect of time of last expression

was observed on the free fatty acid composition of milk from mothers delivering at term. They suggested that a difference in the rates of mammary gland lipogenesis in the preterm and fullterm mammary gland was responsible for these differences.

Variations in maternal hormonal status at different stages of gestation and lactation may account for differences in nutrient composition and the relative rates of synthesis of the constituents of milk. Mothers of preterm infants initiate lactation under different hormonal conditions than those delivering at term. For example, lactation is initiated with lower progesterone and estrogen levels at the start of the third trimester (Erb et al., 1977; Cousins et al., 1977). These differences in hormonal profiles may potentially result in higher levels of prolactin and prolactin receptor levels (Chappell & Clandinin 1985). In turn, these differences in receptor content may result in the altered rates of protein and lipid synthesis observed. This is reflected in the higher levels of mammary lipogenesis reported for some mothers of preterm infants (Chappell & Clandinin , 1982). Findings of increased levels of prostaglandins in colostral preterm milk and changes in milk prolactin concentrations during lactation (Chappell et al., 1983; Healy et al., 1980) also provide evidence that a changing maternal hormonal environment may be responsible for the differences in the nutrient profile of milk observed at different stages of gestation and lactation.

Effect of Maternal Diet

There is some evidence in the literature to suggest that maternal diet plays a role in milk composition (Davila et al., 1983; Finley et al., 1983). However a direct relationship between maternal intake and milk composition has usually been observed when dietary intakes are at extremes (Chappell & Clandinin 1984). For example, inadequate maternal caloric intake during and before the onset of lactation have been associated with lower milk yields and fat concentrations (Lammi-Keefe & Jensen 1984). At 'normal' maternal intakes the effect of diet on milk composition, with a few exceptions, is not significant. Changes in maternal intakes of linoleic acid are associated with changes in milk linoleic acid content (Lammi-Keefe & Jensen 1984). Levels of trans fatty acids in human milk reflect short and long term maternal intake (Chappell et al., 1985). These results indicate that a direct role of maternal diet on milk composition is unlikely. Further research is required to establish the effect of maternal diet on the subcellular control of lactation.

C. The Role of Prolactin in Lactation

Prolactin stimulates the initiation of lactogenesis in the mammary gland by stimulating DNA replication, gene transcription and RNA synthesis (Kelly et al., 1984). The mechanism by which prolactin exerts these effects in not clear. However it has been shown that initiation of prolactin action occurs upon binding of prolactin to basal plasma membrane receptors (Frantz et al., 1974; Kelly et al., 1983).

Properties of the Prolactin Receptor

Prolactin receptors have been isolated from plasma membranes (Frantz et al., 1974: Kelly et al., 1983), microsomal membranes (Ashkenazi et al., 1987; Bohnet et al., 1976; Djiane et al., 1977; Dunaif et al., 1982; Kelly et al., 1974; Posner et al., 1974; Sakai & Ike, 1987; Shiu & Friesen, 1974) and cytosolic regions of the cell (Amit et al., 1984; Ymer & Herington, 1986). Membrane prolactin receptors have been isolated, purified and characterized in several tissues such as the mammary gland, liver, ovary, and testes (Al-Timimi et al., 1987; Amit et al., 1984; Barash et al., 1983; Bramley et al., 1987). The characterization of the membrane receptors have been studied most extensively in rabbit liver and mammary gland (Kelly et al., 1984). The rabbit is the most useful species to study the role of prolactin in lactation because it is the primary hormone involved in lactation in this specie (Shiu & Friesen, 1980). The prolactin receptor appears to be a glycoprotein (Friesen, 1979) with a molecular mass ranging between 43 kdaltons and 200 kdaltons (Dusanter-Fourt et al., 1987; Katoh et al., 1985; Shiu & Friesen 1974). Receptor purification and techniques for estimation of molecular weight varied in these studies which may account for the large range of molecular weights reported in the literature. For example, the use of gel chromatograpy of Triton X-100 solubilized prolactin receptors resulted in molecular mass estimations ranging from 100-200 kdaltons (Shiu & Friesen, 1974). In contrast, molecular mass estimation of prolactin receptors solubilized with zwitterionic detergents ranged between 37 and 55 kdaltons (Liscia & Vonderhaar, 1982; Church & Ebner, 1982: Koppelman & Dufau, 1982; Sakai et al., 1986).

Sakai and Ike (1987) recently isolated two independent prolactin binding subunits with molecular masses of 36.8 and 83.2 kdaltons (predominant, high K_d) in the microsomal membrane of the rabbit gland.

Prolactin Binding

Accurate estimates of prolactin receptor content in target tissue depends upon the ability to measure specific binding of prolactin to its receptor. The most commonly used method to study prolactin receptor binding is to measure **in vitro** the interaction of radiolabelled prolactin with prolactin receptor preparations (Shiu, 1974). Specific binding of labelled prolactin to its receptor represents the difference in the amount of labelled prolactin bound in the presence (nonspecific binding) and absence (total binding) of excess cold prolactin. Specific binding of prolactin has been measured over a range of prolactin concentrations (0-2000 ng/mL) in a variety of tissues and species (Posner et al., 1974). Binding of prolactin to membrane receptors is saturable (Buntin & Ruzycki, 1987), site specific (Roy et al., 1987; N'Guema et al., 1986; Posner et al., 1974; Bramley et al., 1987) and slowly reversible (Kelly et al., 1983; Van der Gugten et al., 1980).

Determination of receptor content in target tissues and characterization of binding affinity is accomplished using Scatchard Analysis (Scatchard, 1949). In this analysis, the ratio of receptor bound hormone to free hormone is plotted against the concentration of bound hormone over a range of cold hormone (free). A linear slope reveals a single class of

receptor type, while a curvilinear slope represents the presence of one or more receptor types. The slope of a linear Scatchard plot depicts the binding affinity constant and the intercept on the abscissa, the total number of binding sites in a receptor preparation (Scatchard, 1949).

Using Scatchard Analysis, it has been demonstrated that a single class of low capacity/high affinity prolactin binding sites exist in plasma membrane (Frantz et al., 1974; Kelly et al., 1983), microsomal membrane (Necessary & Ebner, 1983; Ashkenazi et al., 1987; Bramley et al., 1987; Mitano et al., 1986) and cytosolic fractions (Ymer et al., 1987) of several tissues. Kelly and coworkers (1983) demonstrated that plasma membrane prolactin receptors in rat liver and rabbit mammary gland were lower affinity sites than the microsomal binding sites indicating that receptors with lower affinity at the cell surface are involved in the initiation of prolactin action. The isolation and characterization of cytosolic binding sites raise interesting questions regarding the mechanism of prolactin action. The binding capacity and affinity of membrane sites were also demonstrated to be approximately twice and one sixth of the cytosolic sites respectively. These differences in binding properties of the different receptor types suggest that subcellular location and the distribution of receptors between peripheral and intracellular locations may have important implications for cell function.

D. Regulation of Prolactin Receptors

Although many hormones modify the numbers of prolactin receptors in the mammary gland (Friesen 1979; Sakai & Banerjee, 1979; Bohnet et

al., 1977; Guillaumot et al., 1986), prolactin appears to play the principal role in the regulation of its receptor (Barash et al., 1983; Djiane et al., 1979; Soltysiak & Fellows, 1983). Prolactin can decrease the concentration of its binding sites in a rapid and reversible manner (Barash et al., 1986; Djiane et al., 1979) or increase the number of binding sites over a longer period of time (Posner et al., 1975; Amit et al., 1984; Hughes et al., 1982). The most dramatic change in receptor number occur in the mammary gland during pregnancy and lactation. Receptor levels increase at a slower rate during the first and second trimester of pregnancy and then stabilize or decrease slightly (N'Guema et al., 1986; Djiane et al.. 1977) during the third trimester. High levels of placental-luteal hormones in the latter stages of gestation are thought to inhibit the synthesis of mammary prolactin receptors (Djiane et al., 1977). At parturition levels of mammary prolactin receptors dramatically increase, coincident with a drop in the concentrations of progesterone and estrogen.

Several hormones regulate prolactin action in the mammary gland. These include progesterone (inhibitory), the glucocorticoids (synergistic), estradiol, lutenizing hormone, growth hormone, insulin and the thyroid hormones (Kelly et al., 1984). Prolactin binding can be reduced by estradiol administration (Bohnet et al., 1976), changes with the estrous cycle (Guillaumot et al., 1984) and can be reduced by ovarectomy indicating that estradiol plays a direct role in the physiology of prolactin function. Lutenizing hormones potentiate the effect of estradiol on prolactin action (Guillaumot et al., 1986). Growth hormone mimics prolactin action in the mammary gland. This is not surprising since they share similar molecular

structures and lactogenic properties. Dual regulation of prolactin binding sites by growth hormone and prolactin in rat hepatocytes (Barash et al., 1988) also demonstrates the extent to which growth hormone affects prolactin action. Insulin and the thyroid hormones can be either stimulatory or inhibitory with respect to prolactin action, depending upon the dose utilized (Kelly et al., 1984).

Effect of Membrane Composition on Prolactin Action

The current model of membrane structure proposes that globular proteins are embedded to varying degrees in a lipid bilayer (Singer & Nicholson, 1972). Diet induced changes in the composition of the membrane lipid bilayer have been shown to alter membrane fluidity, resulting in alteration of various membrane associated functions (Clandinin et al., 1985). Diet induced changes in hormone receptor mediated functions have also been shown. Although little research has been done for prolactin, there is some evidence to suggest that changes in membrane lipid composition affect the function of the receptor. In the rat model, changes in total dietary fat and polyunsaturated fat levels in N-methyl-N-nitrosurea initiated mammary and hepatic tumors were associated with alterations in specific binding of prolactin (Cave & Jurkowski, 1984). Reduction in prolactin binding occurred when the dietary polyunsaturated levels fell below three percent suggesting that an unknown level of lipid desaturation must be reached before changes in membrane fluidity result in optimal levels of prolactin binding. Changes in membrane fluidity have been associated with changes in prolactin binding (Bhattacharya & Vonderhaar, 1981; Dave et al., 1985) in

an age dependent manner (Dave & Witorsch, 1983; Dave & Witorsch, 1984). Prolactin appears to increase membrane fluidity upon binding to plasma membrane receptors (Dave & Witorsch 1985) resulting in an increase in the number of available binding sites. Prolactin may mediate these increases in membrane fluidity by stimulating prostaglandin synthesis (Dave et al., 1982) resulting in changes in the lipid microenvironment surrounding the prolactin receptor. These studies provide compelling evidence of a relationship between membrane composition and the function of the prolactin receptor. However further research is required to outline the effect of diet on prolactin action at the cellular level.

E. Mechanism of Prolactin Action

Although it is known that prolactin binding to plasma membrane receptors results in the initiation of lactation, the subsequent molecular events remain unclear. There are two schools of thought in the literature regarding the mechanism of prolactin action. The first is that a classical second messenger system exists and the second is that prolactin mediates its effects on the mammary cell through a direct internalization of the prolactin receptor complex at the cell surface.

Second Messenger Mechanism

There is some **in vitro** evidence in the literature that supports the idea that prolactin exerts its effects through a classical second messenger system. Dusanter-Fourt and coworkers (1984) demonstrated that bivalent and monovalent fragments of anti-prolactin receptor antibodies can mimic

prolactin effects on casein gene expression and on thymidine incorporation into DNA in the rabbit mammary gland. Moreover these antibodies were shown to be equipotent with prolactin in inducing changes in receptor numbers in the membrane preparations (Rosa et al., 1982; Dusanter-Fourt et al., 1984). Crude microsomes from lactating rabbit mammary glands when added directly to isolated mammary nuclei were shown to release a factor capable of stimulating B-casein gene transcription (Teyssot et al., 1981). These findings indicate that internalization and down regulation of receptor numbers are not directly related to prolactin action on casein or DNA synthesis in the mammary gland.

Attempts to identify the second messenger of prolactin action has had limited success. Activation of the sodium-potassium ATPase, flux of calcium ions, activation/inhibition of cyclic nucleotides, an enhanced rate of prostaglandin biosynthesis mediated by a stimulation of phospholipase A_2 activity and a stimulation of polyamine synthesis (Rillema, 1980; Shiu & Friesen, 1980) have been identified as potential second messengers of prolactin action in the mammary gland. The most compelling evidence for the existence of a second messenger lies in the role of prostaglandins on prolactin action. For rodent models it has been observed that prolactin action is associated with the stimulation of phospholipase A_2 activity and the release of arachidonic acid from membrane phophatidylcholine (Rillema, 1980) resulting in the synthesis of prostaglandins. These changes in prostaglandin levels have been associated with changes in mammary RNA synthesis and membrane fluidity (Dave et al., 1982: Rillema, 1980) suggesting that prolactin may regulate receptor numbers and hence influence prolactin

action through this mechanism. Prolactin action has also been associated with phosphoinositol hydrolysis in cultured mouse mammary explants (Etindi & Rillema, 1988). It is possible that this hydrolysis could stimulate the synthesis and release of prostaglandins, 1,2-diacylglycerol and calcium ions from the plasma membrane and that these factors could work in concert or independently to stimulate casein synthesis in the nucleus of the mammary cell.

Intracellular Mechanism of Prolactin Action

Intracellular binding sites for prolactin action have been identified in several tissues using a variety of analytical techniques (Katoh et al., 1986: Shiu & Friesen, 1976; Dunaif et al., 1982). Prolactin binding sites have been identified in the Golgi fractions (microsomal) (Dunaif et al., 1982; Shiu & Friesen, 1976) and cytosolic fractions (Ymer et al., 1987) of the mammary gland. Studies of these binding sites using antibody techniques indicate that these sites share common but not identical, immunological characteristics (Ymer et al., 1982; Berthon et al., 1987; Shiu & Friesen 1976; Katoh et al., 1985). The specific roles of these receptors in prolactin action have not been elucidated. However there is some evidence to suggest that these intracellular binding sites may mediate some of the actions of prolactin within the cell. For example, it has been demonstrated that upon binding to plasma membrane receptors radiolabelled prolactin is internalized within the mammary cell and directed to microsomal and lysozomal compartments of the cell (Basset et al., 1984; Coslow & McGuire, 1977; Ferland et al., 1984; Giss & Walker, 1985) where it binds

to these sites. The significance of these binding interactions are unknown. However, the fact that internalized prolactin remains intact and shows signs of immunoreactivity suggests that binding of prolactin to microsomal fractions may have a direct effect on milk synthesis in the cell. The presence of bioactive prolactin capable of binding to prolactin receptor preparations and the presence of a prolactin binding protein in milk secretions (Gupta, 1983; Waters et al., 1980; Clandinin et al., 1986; Taketani & Mizuno, 1985) also provides indirect evidence that an intracellular mechanism for prolactin action exists in the mammary gland.

F. Prolactin and Prolactin Receptor Content of Milk

Milk contains a large number of bioactive substances. These include a wide variety of hormones (prolactin), enzymes and growth factors (Strbak, 1985; Gupta, 1983). Prolactin levels in milk appear to approximate maternal serum levels (Malven & McMurtry 1973; Malven & McMurtry, 1974; Mulloy & Malven, 1979). Milk levels increase initially in the first three days following delivery and then fall sharply (Grosvenor & Whitworth, 1983; Healy et al., 1980). These changes are paralleled by dramatic changes in the nutrient composition of milk (Kulski & Hartman, 1983; Kulski et al., 1977; Healy et al., 1980) and in prolactin receptor content (Waters et al., 1980) over the first two weeks of lactation. Changes in milk prolactin receptor content reflect the changes in mammary gland content (Waters et al., 1980). These changes in milk content at different stages of lactation suggest that a direct relationship exists between mater-

nal hormonal environment and the partitioning of nutrients between mother and infant.

The mechanism by which prolactin enters milk is not known. Prolactin may enter the milk through the alveolar cells bound to intracellular proteins (Healy et al., 1980; Grosvenor & Whitworth, 1983) indicating that an intracellular mechanism for prolactin action may exist in the mammary gland. The significance of a changing maternal hormonal environment has important implications for the developing neonate. For example, at the start of the third trimester lactation is initiated with lower circulating levels of progesterone and estradiol resulting in potentially higher prolactin receptor levels and in altered rates of protein and lipid synthesis (Chappell & Clandinin, 1985). Hence the preterm infant is exposed to a different nutrient profile than the fullterm infant (Atkinson et al., 1978; Lemons et al., 1982). The presence of prolactin in human milk may also have short and long term effects on the development of the neuroendocrine system of the infant (Malven, 1983). Milk prolactin may directly influence gonadal and adrenal function in the newborn by contributing to serum pools of prolactin (Grosvenor & Whitworth, 1983). Maternal prolactin may also play a role in fluid and ion absorption in the infant (Malven, 1983).

G. Conclusions

Milk lactated by mothers delivering prematurely differs in macronutrient content when compared with milk from mothers delivering at term (Atkinson et al., 1978; Lemons et al., 1982). Maternal hormonal status may be reponsible for these difference in composition. The mechanism by

which prolactin stimulates milk synthesis is not known. Recent evidence indicates that an internalization of the prolactin-receptor complex at the cell surface initiates the action of prolactin in the mammary gland (Ymer et al., 1987). The presence of biologically potent prolactin and a prolactin binding protein in milk provide indirect evidence that an intracellular mechanism for prolactin action exist in the mammary gland. Assay of these factors in milk at different stages of gestation and lactation may be used to assess the relationship between maternal hormonal status and the nutrient composition of milk. Maternal diet may also interact with these hormonal effects. Dietary fat has been shown to affect receptor mediated functions by altering the fatty acid composition of membrane phospholipids (Clandinin et al., 1985). Further research is required to establish the role of maternal diet on prolactin action at the cellular level.

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II. Research Plan.

A. Rationale

For humans there is little information concerning the intracellular mechanism by which prolactin exerts its effect on the mammary gland. Human milk contains significant quantities of biologically potent prolactin (Gupta, 1983; Healy et al., 1980, Gala et al., 1975) capable of binding to liver prolactin receptor preparations (Taketani & Mizuno 1985) suggesting that sites of action for this hormone may also exist in milk. A prolactin binding protein has also been isolated in milk (Clandinin et al., 1986; Waters et al., 1980). The presence of prolactin and a prolactin binding protein in milk would support the existence of an intracellular pathway for prolactin action in the mammary gland. Measurement and comparison of milk prolactin content and prolactin receptor content with nutrient levels present in the milk from mothers who lactate after different lengths of gestation (delivery at term or at the start of the third trimester) may help explain how changes in maternal hormonal status alters lactogenesis in humans.

B. Statement of Objectives

The objective of this study was to determine if a prolactin binding protein exits in human milk and to isolate, quantitate and characterize the properties of a prolactin binding protein in the milk at different stages of gestation and duration of lactation. Milk samples were collected from mothers that were descriptively similar and producing sufficient volumes

of milk to meet the energy needs of their infant. Milk prolactin, lactose, protein and total fat was quantitated and related to milk prolactin receptor content. The fatty acid composition of microsomal membrane phospholipids was also determined and studied in relation to the binding properties of the prolactin receptor.

C. Hypothesis

It is hypothesized that human milk contains a prolactin receptor that selectively binds human prolactin. If specificity can be demonstrated for this receptor then it can be specifically hypothesized for human milk that

1). Prolactin receptor content of the milk is related to the stage of gestation and lactation and thus to the hormonal status of the mother.

2). Prolactin receptor content is related to the prolactin content of the milk.

3). Prolactin receptor content is related to the total fat, protein, and lactose content of the milk.

4). The properties of prolactin receptor binding are related to the fatty acid composition of the microsomal membrane phospholipids at different stages of gestation and lactation.

D. Chapter Format

The hypotheses proposed were tested in all milks collected. These experiments are organized as thesis chapters. Chapter 1 is a literature review of prolactin binding in various tissues and species. Chapter II outlines the research plan of this project. Chapter III examines the properties of the prolactin receptor in milk at different stages of gestation and lactation (Hypothesis 1). Prolactin binding capacity was examined in relation to prolactin levels present in the milk (Hypothesis 2). Prolactin receptors numbers were examined in relation to the nutrient composition of the milk at these different stages (Hypothesis 3). Chapter IV examines the relationship of the fatty acid composition of microsomal phospholipids to prolactin binding (Hypothesis 4). Chapter V contains a discussion of prolactin receptor properties in relation to all topics of study. General implications that may be drawn from this study will also be discussed in this chapter.

III. PROPERTIES OF PROLACTIN BINDING AT DIFFERENT STAGES OF GESTATION AND DURATION OF LACTATION AND RELATIONSHIPS TO THE NUTRIENT COMPOSITION OF MILK

A. Introduction

Studies have shown that milk lactated by mothers delivering prematurely differs in macronutrient content from milk produced by mothers delivering at term (Chappell & Clandinin, 1982; Atkinson et al., 1978; Lammi-Keefe & Jensen, 1984). These differences are influenced by maternal hormonal status (Chappell & Clandinin, 1984; Kulski & Hartmann, 1983; Healy et al., 1980) and diet (Neville et al., 1983). Prolactin, a key hormone for lactogenesis, has been shown to be present in human milk (Gupta, 1983; Healy et al., 1980; Gala et al., 1975; Taketani & Mizuno, 1985) and the milk of several other mammalian species (Malven, 1983; McMurty & Malven, 1974; Grosvenor & Whitworth, 1983; Mulloy & Malven, 1979). Milk microsomal membrane has also been shown to contain a prolactin binding protein (Clandinin et al., 1986; Waters et al., 1980). The mechanism by which prolactin enters milk is not clear. It has been postulated that prolactin may enter milk through the mammary alveolar cells bound to intracellular proteins (Basset et al., 1984; Dunaif et al., 1982; Ferland et al., 1984; Giss & Walker, 1985). These hormone-protein complexes may represent the internalization of a prolactin-receptor complex at the cell surface or internal sites of prolactin action within the mammary cell (Ymer et al., 1987; Nolin & Witorsch, 1976) indicating that an intracellular mechanism for prolactin action may exist in the mammary gland.

The physiological significance of the presence of prolactin and a prolactin receptor in milk is not known. However, it has been shown in the rat model that milk prolactin levels contribute significantly to neonatal plasma prolactin levels (Grosvenor & Whitworth, 1983) suggesting that maternal prolactin may play a role in the development of the newborn endocrine system. For humans, there is little information regarding the hormonal parameters that influence the partitioning of nutrients between mother and infant under differing maternal hormonal status. The presence of prolactin and a prolactin receptor in milk allows one to assess prior maternal hormonal events leading to lactation. Thus, the following study was designed to assess the effect of changes in maternal hormonal status in lactation by the measurement of prolactin and prolactin receptor levels at different stages of gestation and duration of lactation.

Prolactin receptors were isolated, quantitated and characterized in milk lactated by mothers delivering prematurely (28-34 weeks of gestation) and at term to establish properties of prolactin binding under differing maternal hormonal status. Comparison of prolactin and prolactin receptor levels with the nutrient composition of milk at different stages of lactation was done to assess the effect of maternal hormonal status on the availability of nutrients in milk to the newborn.

B. MATERIALS AND METHODS

Subject Criteria and Milk Collection

Subject selection and milk collection procedures were approved by the University of Alberta Hospital Human Ethics Committee. Milk samples were collected from fifteen mothers delivering at term and fifteen mothers delivering between 28-34 weeks of gestation on day 4, 16 and 37 postpartum. Milk was expressed mechanically from the left breast using a Gerber Precious Care® breast pump during the first early morning expression (6-7 am;T1) and approximately two hours later (8-9 am;T2). The first collection was preceded by a period of approximately six hours since the last time of nursing, 11-12 pm the previous evening. Complete expressions were collected at both times. Volume, time of expression and the time of last nursing or expression were recorded by the volunteer. Milk samples were stored in glass bottles initially at 4°C for less than twenty-four hours until transported on ice to the laboratory. Samples were measured, aliquoted and stored frozen at -70°C until assay.

General information regarding the mothers' background and medical history (course of pregnancy and delivery) were recorded at the time of subject recruitment. Pregnancy and delivery of both preterm and fullterm infants followed a normal course. All mothers were normal healthy individuals producing enough milk to meet their infants' energy requirements. Any mother ingesting medication that would potentially interfere with the normal hormonal environment of pregnancy and lactation (del Poza et al., 1979) was excluded from this study. For example, any mother ingesting

drugs such as chlorpromazine, ergot alkaloids or who had a history of medical illness (diabetes, hypertension) would normally be excluded from this study.

Properties of prolactin binding were determined in pooled preterm (n=5) and pooled fullterm (n=7) milks collected 4, 16 and 37 days postpartum between 6-7 am and 8-9 am. The remaining preterm (n=10) and fullterm (n=10) milk samples were used for determination of prolactin receptor, prolactin and nutrient content at different stages of gestation and duration of lactation. Based upon preliminary analysis, it was determined that 70-80 mls of milk was required to provide sufficient membrane for prolactin binding, fatty acid analysis of membrane phospholipids and protein analysis. In order to fullfill this requirement, milk from two subjects were consistently pooled for the same day and time, on days 4, 16 and 37, to ensure that the total volume of milk in each group exceeded 60 mLs.

The fullterm samples collected on day 4 were very small (less than 20 mLs per expression) making it necessary to pool four subjects for each time of collection. This resulted in two pools of four subjects and one pool of two subjects on day 4 (T1 and T2) for the fullterm group (n=3).

Milk Membrane Isolation

Pooled milk samples (12-15 mLs) were mixed with 20-25 mL of isolation buffer containing 25 mM TRIS-HCl, 10 mM $MgCl_2$ pH 7.5 and centrifuged at 12,700 rpm (20,000 x g at r_{av}) for 30 minutes at 4°C in a Beckman L8-70M Ultracentrifuge and SW 28 rotor (Beckman Instruments Ltd. Palo Alto, California) to remove fat and particulate matter (Waters et

al., 1980). The defatted supernatant was diluted with 5-10 mLs isolation buffer and centrifuged at 27,500 rpm (100,000 x g at r_{av}) for 90 minutes at 4°C in a Beckman SW 28 rotor. The supernatant was removed and stored at -70°C. The 27,500 rpm (100,000 x g_{rvs}) pellet was resuspended in 2 mLs of 5M $MgCl_2$ containing 0.1M TRIS-HCl buffer (pH 7.5) at 23°C. After 5 minutes the sample was diluted with 30-35 mLs of isolation buffer and centrifuged at 27,500 rpm (100,000 x g r_{av}) at 4°C for 60 minutes in the same rotor to sediment the membranes. The supernatant was aspirated, the pellet resuspended in 30-35 mLs of isolation buffer and then recentrifuged at 27,500 rpm for 60 minutes at 4°C to remove excess 5M $MgCl_2$. The membrane pellet was collected, resuspended in 600-800 µl of isolation buffer, and stored at -70°C with pellets prepared from aliquots (12-15 mLs) of the same pool of milk until assay.

Prolactin Binding

Milk microsomes treated with 5M $MgCl_2$ were incubated with ^{125}I-human prolactin (New England Nuclear, Boston MA US) as described by Shiu and Frisen (1974) and Waters et al. (1980). Membrane protein (300 µg) was incubated in 12x75 mm glasstubes with 100 µl of ^{125}I-hPRL (0.045 µCi/tube or 1-2 ng/tube) in the absence or presence of varying levels of unlabelled prolactin (0-40 ng, 15 µg/mL, Friesen:Batch 84-7-20) in the assay buffer (25 mM TRIS-HCl, 10 mM $MgCl_2$,0.1% (w/v) BSA pH 7.5) in a final volume of 0.5 mL at 23°C for 16-18 hours. Prolactin binding was terminated by the addition of 1 mL of cold assay buffer and centrifugation in a Beckman J2-21 centrifuge (JA-21 rotor) at 5000 rpm (3000 * g_{rav} for

30 minutes at 4°C. The resulting supernatant was decanted and tubes were inverted and drained on absorbent paper. The pellets were counted in a Beckman LS-8000 gamma counter (Radiation Services, Pharmaceutical Sciences, University of Alberta) with a counting efficiency of 75%. Specific binding was determined by subtracting the counts bound in the presence of excess (1 ug) unlabelled hPRL (nonspecific counts) from counts bound in the absence of excess unlabelled hormone (total binding). The nonspecific counts were 45-65% of total counts bound. Scatchard analysis of every sample was performed with 6-11 point duplicate determinations over the range of 0-40 ng added cold prolactin. The number of points used in each analysis depended upon the microsomal membrane yield from the milk which varied among samples and the level of intraassay variability. Scatchard analysis was used to calculate the binding capacity and dissociation constant by linear regression ($p<.05$).

Prolactin Determination

The prolactin content of milk was assayed using the human prolactin radioimmunoassay kit distributed by NIAMDD. The methods used were similar to those suggested by NIAMDD and Mulloy and Malven (1979). Pooled milk samples were diluted 1:2.5 in the assay buffer (0.01M phosphate buffered saline conatining 0.1% (w/v) sodium azide, 0.15M NaCl, 1% (w/v) BSA pH 7.5) and centifuged at 5000 rpm (3000 x g r_{av}) for 30 minutes at 4°C in a Beckman J2-21 centrifuge. At the end of this

centrifugation, the lipid layer was removed and the defatted diluted milk samples stored frozen at -20°C until assay.

Defatted milk samples (50 µl) or standard human prolactin antigen (NIAMDD-hPRL-RD-1) (100 µl) of varying concentrations (1-500 ng/mL) were added to 200 µl of human prolactin antiserum (NIAMDD-anti-hPRL-3; 1:400,000), 100 µl of ^{125}IhPRL (0.0045 µCi/tube; 0.1 ng/tube obtained from New England Nuclear, Boston, MA, USA) and 300-350 µl of 1% (w/v) BSA-PBS for a final volume of 700 µl. After 48 hours at 4°C, 200 µl of goat-antirabbit gammaglobulin (Sigma Chemical Company, St. Louis, Mo, USA) diluted 1:25 was added to each tube. The tubes were incubated for another 48 hours at 4°C. At the end of this period one mL of cold PBS (4°C) was pipetted into each tube and the tubes centrifuged at 5000 rpm (3000 x g r_{av}) for 30 minutes at 4°C in a Beckman J2-21 centrifuge (JA-2 rotor). The supernatant was decanted and the precipitate counted in a Beckman LS-8000 gamma counter. Blanks contained isotope, assay buffer and antirabbit gamma globulin. The validity of milk prolactin estimates was assessed by recovery experiments in which 0.1-10 ng/mL of standard human prolactin was added to a standard fullterm milk (collected and pooled 4, 16 and 37 days postpartum between 6-7 am and 8-9 am (n=7)). The immunological specificity of milk prolactin measurement was assessed by comparision of displacement of ^{125}I-human prolactin by increasing concentrations of human prolactin antigen with displacement caused by varying levels of milk prolactin. Lack of parallelism between the two curves indicated a lack of immunological specificity in this assay. The precision of the assay was determined by repeated assay of standard pools of

fullterm and preterm milk (collected in same manner as fullterm pool (n=4) within and between assays. All prolactin determinations were duplicate determinations.

Protein Determination

Pooled milk samples were diluted 1:2.5 in 25 mM TRIS-HCl, 10 mM $MgCl_2$ pH 7.5 and centrifuged at 5000 rpm (3000 x g r_{av}) for 30 minutes at 4°C in a Beckman JA-21 rotor. The lipid layer was discarded and the defatted supernatant stored frozen at -20°C until assay. Protein content was determined on aliquots of this supernatant using the method of Lowry et al. (1951).

Total Fat Determination.

Milk lipids were extracted using an adapted Folch procedure described by Chappell et al. (1985). Pooled milk samples (1 mL) were homogenized using a Polytron P-10 (Brinkman Instruments, Wesbury), added to 18 mL Chloroform (C)/Methanol (M) (1:1) containing 100 µl of internal standard ($C_{19:0}$ 30 mg/mL) and vortexed for two minutes. Samples were filtered gravimetrically through Whatman #43 filter paper into a 125 mL separatory funnel. Fifty mLs of chloroform/methanol 2:1 was added into each separatory funnel and inverted to mix. Fifteen mL of 0.05% (w/v) $CaCl_2$ was added to each seperatory funnel, mixed and stored at 4°C for at least eight hours. The bottom layer was collected in tared 50 mL culture tubes and dried at 40°C under vacuum (Model RH 12-29 Speed Vac Concentrator Centrifuge; Savant Instruments Inc., Ont.) for 5-6 hours.

The samples were weighed, flushed with N_2, capped with teflon lined caps and stored at -70°C.

Lactose Determinination

The lactose content of milk was assayed according to a modified method of Kotler et al. (1981). Pooled milk samples were diluted 1:50 in deionized water and centrifuged at 5000 rpm (3000 x g r_{av}) for 30 minutes at 4°C in a Beckman JA-21 rotor. The lipid layer was discarded and the defatted supernatant stored frozen at -20°C until assay. Defatted milk samples (50 µl) or 50 µl of standard β-lactose (obtained from Sigma Chemical Co. St. Louis Miss. USA) of varying concentrations (0-1.27mM) were added to 100µl of β-galactosidase (80 µg/µl reaction mixture; EC 3.2.1.23; obtained from Sigma Chemical Co. St. Louis. Miss. USA) and 1 mL of incubation buffer containing 0.1M sodium phosphate (pH 7.4). The tubes were incubated for two hours at 30°C in a shaking water bath. The free glucose content was then determined by adding 5 mLs of PGO (peroxidase-glucose oxidase) enzyme-colour (o-dianisidine dihydrochloride) solution (Glucose diagnostic kit obtained from Sigma Chemical Co. St. Louis Miss. USA) to each tube. The colour was allowed to develop at room temperature for 45 minutes and analyzed at 420 nm. Corrections for blanks and the presence of endogenous glucose in milk were made. A standard glucose curve (0-1.24 mM) was run with each batch of milk samples to assess cleavage of standard lactose solutions.

Statistical Analysis

The effect of gestational age and duration of lactation on the prolactin concentration, prolactin receptor and nutrient composition of milk was assessed by Least-Squared Analysis of Variance procedures (Harvey, 1975). Regression analysis was used to assess the relationship between maternal prolactin status (as reflected by prolactin receptor content and prolactin concentration in milk) and the nutrient composition of milk.

C. RESULTS

Characteristics of Subjects

The average age of the mothers delivering prematurely (28-34 weeks of gestation) and at term was not significantly different (28.5;n=10 and 25.9;n=10 years, respectively). The mean gestational age, weight and length of the preterm infants (31.8 weeks, 1806 gms and 42.3 cm respectively) was significantly lower than the fullterm infants (39.9 weeks, 3341 gms and 49.3 cm repectively) (Table III-1) ($p<.05$).

The volume of milk produced between 6-7 am(T1) and 8-9 am(T2) on each collection day remained unchanged over the duration of the study in each group (52.8 and 38.9 mL, respectively). No significant difference in average milk volume was apparent between fullterm and preterm samples over the course of the study (49.4 and 42.3 mLs, respectively). Pooling of milk was associated with significant differences in mean volume between groups and between times of collection over the duration of the study.

The overall mean for pooled volume (mean ± SD) for fullterm and preterm milk was 94.9 ± 38.7 mL (n=26) and 74.9 ± 24.6 mL (n=30), respectively. Mean pooled volume (mean ± SD) for milk expressed between 6-7 am and 8-9 am was 99.5 ± 34.5 mL (n=28) and 68.9 ± 18.3 mL (n=28), respectively (Table III-2) ($p<.05$).

Subcellular Location of Milk Prolactin Receptor

In a preliminary study, total binding of ^{125}I-human prolactin to various subcellular fractions of milk was measured in preterm (n=5) and fullterm (n=5) milk samples collected sixteen days postpartum between 6-8 am. Analysis of the binding of ^{125}I-human prolactin to the 20,000 x g pellet, 20,000 x g supernatant and 100,000 x g microsomal pellet showed that 70-90% of the total binding of ^{125}I-human prolactin was located in the 100,000 x g supernatant and 100,00 x g microsomal fractions (Table A1.1). Treatment of preterm and fullterm milk microsomes with 2.5M and 5M $MgCl_2$ resulted in a 2-4 fold and a 10-14 fold increase in prolactin binding, respectively. Binding of ^{125}I-human prolactin to fullterm milk was significantly higher than preterm milk in all fractions tested (Table A1.1) ($p<.05$). Binding of ^{125}I-human growth hormone in preterm and fullterm milk was significantly lower than ^{125}I-human prolactin in all fractions tested displaying the selectivity of prolactin binding in human milk (Table A1.II) ($p<.05$). Treatment of fullterm milk microsomes with 2.5M and 5M $MgCl_2$ resulted in a 15 fold increase in total growth hormone binding. No apparent difference in total binding of ^{125}I-human growth hormone to treated and untreated preterm milk microsomes was observed.

Table III-1. Subject Descriptive Data

Group	Age of Mother (years)	Sex of Infant	Weeks of Gestation	Weight of Infant (g)	Length of Infant (cm)
Preterm	28.5 ± 4.1[a]	7F 3M[a]	31.8 ± 2.2[a]	1806 ± 566[a]	42.3 ± 5[a]
Fullterm	25.9 ± 4.2[a]	4F 6M[a]	39.9 ± 1.5[b]	3341 ± 488[b]	49.3 ± 41[b]

F refers to female infants, M to male infants. Values are group means ± SE (n = 10). Values without a common superscript are significantly different ($p < .05$).

Table III-2. Volume of Milk Collected at Different Stages of Gestation and Lactation.

Group	Day 4		Day 16		Day 37	
	T1	T2	T1	T2	T1	T2
Preterm	43.8 ± 18.5[a]	36.2 ± 19.5[a]	52.6 ± 24.1[a]	41.4 ± 14.2[a]	43.9 ± 21.5[a]	35.7 ± 20.7[a]
Preterm (pooled)	87.5 ± 15.9[b]	72.4 ± 14.1[b]	105.1 ± 41.4[b]	82.8 ± 24.3[b]	87.7 ± 14.3[b]	71.4 ± 27.6[b]
Fullterm	33.8 ± 26.0[a]	32.7 ± 20.1[a]	66.1 ± 29.6[ab]	41.8 ± 29.8[ab]	76.3 ± 46.9[ab]	45.6 ± 23.7[ab]
Fullterm (pooled)	112.7 ± 32.6[b]	109.1 ± 49.3[b]	132.1 ± 47.9[b]	83.5 ± 24.3[b]	137.3 ± 56.5[b]	91.1 ± 26.5[b]

Milk was collected from mothers delivering prematurely (28-34 weeks) and at term on three different days (4, 16 and 37) after delivery. Milk was manually expressed from the left breast at 6-7 am (T1) and 8-9 am (T2) on these days. Milk from two subjects was pooled for the same day and time on days 4, 16 and 37 for both groups. Two sets of four subjects were pooled for the fullterm group on day 4 (T1 and T2). Unpooled values are group mean ± SE (n = 10). Pooled values are group mean ± SE (n = 5) for day 16 and 37 Preterm. Day 4 values are group mean ± SE (n = 3; n = 5). All values are compared with each other. Values without a common superscript are significantly different ($p < .05$).

Analysis of specific binding of ^{125}I-human prolactin in subcellular fractions of fullterm milk (n=1) was measured to assess the location of the prolactin receptor in human milk. Binding of prolactin to milk microsomes treated with 5M $MgCl_2$ was significantly higher ($p<.05$) than binding in the 20,000 x g pellet and 100,000 x g supernatant in agreement with results reported by Waters et al., (1980). Specific binding was not detected in the 20,000 x g supernatant (Table A1-III). Characterization of prolactin binding in milk microsomes treated with 5M $MgCl_2$ was undertaken to establish the validity of Scatchard analysis to determine receptor numbers and dissociation constants in preterm and fullterm milk over the duration of lactation.

Molecular Weight Determination

The molecular mass of the milk microsomal prolactin receptor was determined by the fractionation of the ^{125}I-human prolactin-receptor complex on a calibrated Sephadex G-100 column. A plot between elution volume (mL) and the logarithm of molecular mass of marker proteins was used as a standard curve for this determination (Figure A1). The ^{125}I-human prolactin receptor complex eluted near the column void volume indicating a molecular mass of 140,000 and 117,000 daltons for the prolactin-receptor complex and free receptor, respectively.

Properties of Prolactin Binding

Optimal conditions for prolactin binding to milk microsomes treated with 5M $MgCl_2$ was determined in pooled preterm (n=4) and fullterm (n=7)

milk samples. These samples were collected 4, 16 and 37 days postpartum between 6-7am and 8-9am. Preterm and fullterm samples were pooled for each day and time to provide homogeneous samples representative of all binding characteristics over the course of the study.

Specific binding was examined over a range of ^{125}I-human prolactin concentrations (0.1-10 ng/mL) to determine the level of radioligand necessary for optimal conditions for binding (data not illustrated). A level of 2-3 ng/mL was chosen for all binding analysis as marked variations in specific binding occurred at higher concentrations. Three different batches of ^{125}I-human prolactin were used to analyze all milk samples to minimize errors due to decreases in biological activity of the radioligand with time. No significant difference in specific binding was detected between batches of radioisotope (Table AII).

A comparison of specific binding between membranes treated with and without detergent at varying concentrations of ^{125}I-human prolactin was done to assess the effect of CHAPS (3-[3-chloamidoprophyl) dimethylammonio]-1-propanesulfonate) treatment (Table AIII). No significant difference in specific binding was detected between treated and untreated membranes at low concentrations of ^{125}I-human prolactin (1.6-3 ng/mL). However, at higher concentrations of ^{125}I-human prolactin (5 ng/mL), specific binding in treated membranes) was significantly higher ($p<0.05$) than in untreated membranes. As this difference was only detected at high radioligand concentrations, solubilization of milk micorsomes with CHAPS was not done in this study.

Membrane Protein

Total, nonspecific and specific prolactin binding increased with increasing levels of membrane protein (Figure III-1). Specific binding was not distinguishable from nonspecific binding at membrane protein concentrations less than 200 μg/mL and was maximal at concentrations above 500-600 μg/mL. Therefore, a membrane concentration of 600 μg/mL was used for all binding assays. The nonspecific binding of the system was variable between samples. Nonspecific and specific binding represented 35-45% and 45-65% of the total counts bound, respectively. Total binding represented 15-25% of the total counts added.

Effect of time, temperature and pH on prolactin binding

Specific binding increased with time of incubation at 23°C, and plateaued after 15-18 hours (Figure III-2). Specific binding was markedly reduced at 4°C over the incubation period. Binding was highest at pH 6.5 to 7.5 (Table III-3). High and low pH resulted in minimal binding. Subsequent binding studies were carried out at membrane protein concentration of 600 μg/mL at 23°C, pH 7.5, and an incubation period of 15-18 hours.

Reversibility and Specificity

The reversibility of binding to the prolactin receptor was assessed by measuring displacement of ^{125}I-human prolactin with excess cold prolactin (1 μg) over time. The tubes were preincubated with hot prolactin for 16-18 hours to ensure equilibrium conditions for binding. After eight

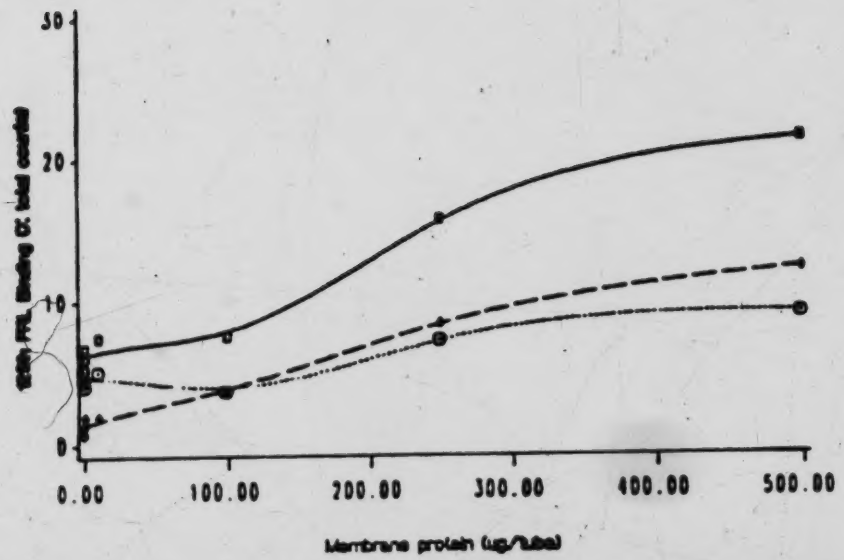

Figure III-1. Changes in mean total, nonspecific and specific binding of ^{125}IhPRL to milk microsomal membranes treated with 5M $MgCl_2$ as a function of membrane protein concentrations.

^{125}Ih PRL (25,000 cpm, 0.6 ng) was incubated in the presence and absence of cold prolactin (2 ug/ml) with varying amounts of milk microsomal membrane treated with 5M $MgCl_2$. Microsomal membranes isolated from a fullterm milk collected 4, 15 & 37 after delivery between 6-7 am & 8-9 am. The non-specific bound prolactin was 45-65% of the total counts bound. Total bound prolactin was 15-25% of the total counts added. Values are means ± SE of duplicate determinations. Total Bound Non-Specific Binding ⊕ ⊕-⊕ Specific Binding ⊕-⊕-⊕

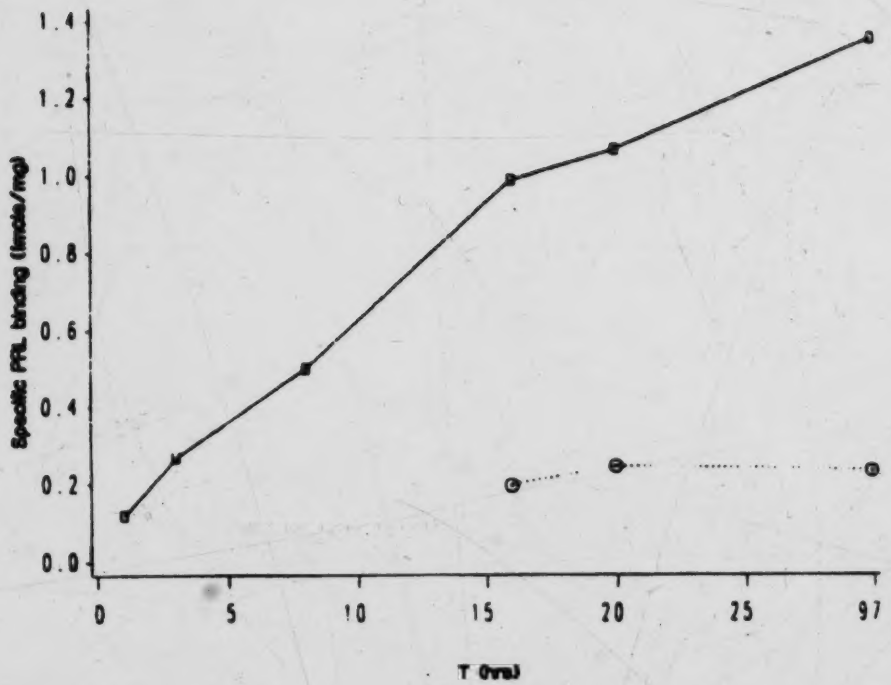

Figure III-2. Effect of Time and Temperature on Prolactin Binding.

The amount of ^{125}Iprolactin bound over time at 23 °C and 4 °C. Milk microsomes were prepared from pooled fullterm milk (n = 7) collected on 4, 16 and 37 days postpartum between 6-7 am and 8-9 am. Values are means of duplicate determinations. T = 23 °C ⊖—⊖—⊖ T = 4 °C ⊙·⊙·⊙

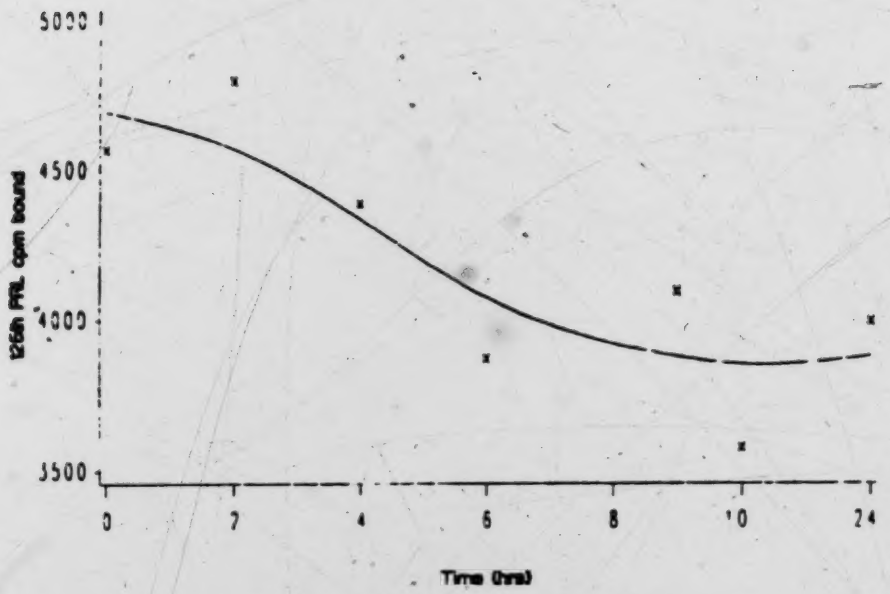

Figure III-3. Reversibility of prolactin binding to 5M $MgCl_2$ treated microsomes.

The amount of ^{125}IhPRL displacement (50,000 cpm, 1.6ng) by 1 ug cold prolactin over time was measured in microsomes prepared from a pool of six fullterm milk samples (collected 4, 16 and 37 days after delivery between 6-7 am and 8-9 am). To ensure equilibrium conditions, membranes were incubated with ^{125}IhPRL for 16-18 hours before the addition of excess prolactin. Values represent mean ± SE of duplicate determinations.

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hours, fifty percent of the bound prolactin was displaced by cold prolactin (Figure III-3) displaying the reversibility of prolactin binding.

The specificity of prolactin binding was examined by measuring the displacement of ^{125}I-human prolactin by increasing concentrations of a variety of cold competitors (Figure III-4). The most effective inhibitor was human prolactin followed by porcine prolactin and human growth hormone which displaced bound ^{125}I-human prolactin with similar potencies. Displacement of ^{125}I-human prolactin by human prolactin was significantly greater ($p<0.05$) than human growth hormone and porcine prolactin at the lower range of hormone levels (1-15 ng) examined, displaying the greater affinity of the receptor for human prolactin. No significant displacement was produced by insulin at the higher concentrations (2 μg/mL), although minimal displacement was observed at the concentrations (20-80 ng/mL). This displacement profile displays the specificity of this receptor preparation for lactogenic hormones.

Binding Capacity, Affinity and Saturability

Scatchard Analysis (Figure III-5) was used to determine the binding capacity and dissociation constant (K_D) of the milk prolactin receptor (Scatchard, 1949). Specific binding was measured over a wide range of prolactin concentrations (0-2000 ng/mL) to determine if binding was saturable (Figure III-6). Maximal binding of prolactin to fullterm and preterm milk microsomes was acheived at prolactin concentrations of 60 and 80 ng/mL, respectively. Therefore, Scatchard analysis was performed

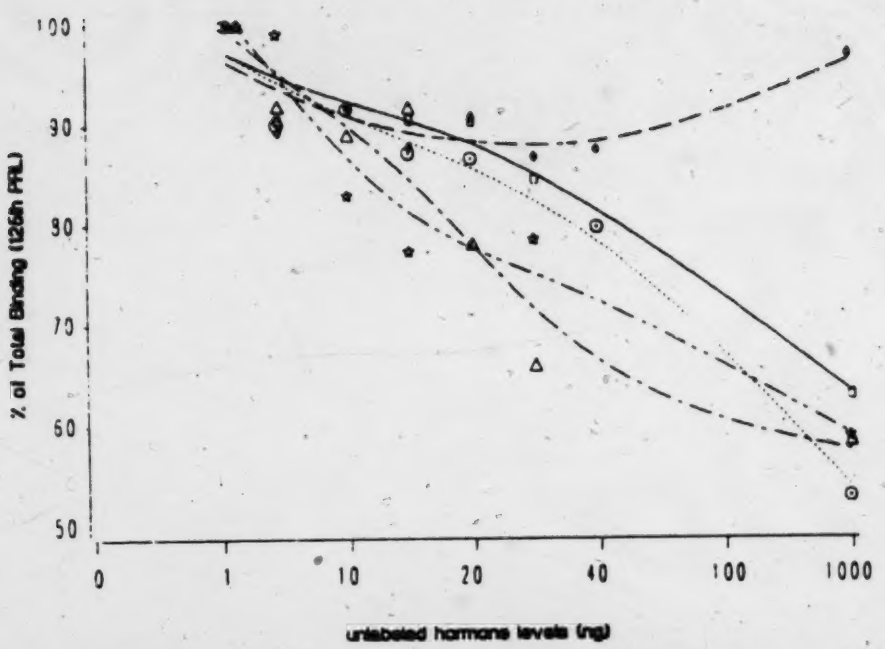

Figure III-4. Competitive inhibition of bound ^{125}Ih PRL to milk microsomal membranes treated with 5M $MgCl_2$ by unlabeled hPRL, human growth hormone (hGH), porcine prolactin (pPRL) and porcine insulin.

Values represent means of duplicate determinations at each level of competing hormones. Two concentrations of ^{125}IhPRL were used to determine specificity of binding. Human prolactin , pPRL, and porcine insulin were incubated with ^{125}IhPRL (1.2 ng/ml, 55,000 cpm) and 300 ug of membrane protein. Human growth hormones and human prolactin was incubated with ^{125}IhPRL (4.4 ng/ml, 62,000 cpm) and 300 ug of membrane protein. Membranes were prepared from pooled fullterm milk samples (n = 7) collected at 4, 16 and 37 days postpartum between 6-7 am and 8-9 am. hPRL (1.2 ng/ml) ●—●—● pPRL ⊖ ⊙ hinsulin ♦—♦—♦ hGH △—△ -△ hPRL (4.4 ng/ml) ★ ★ ★

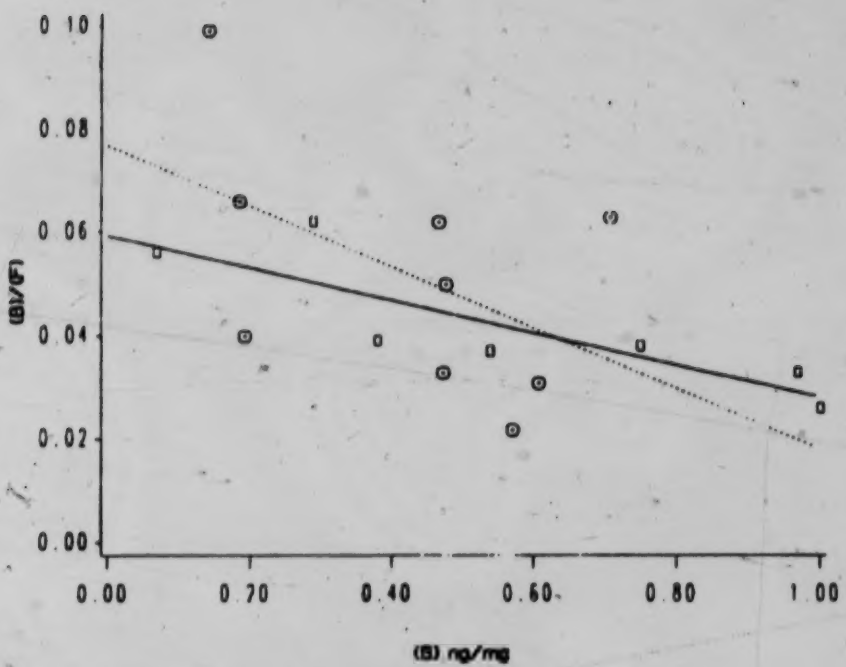

Figure III-5. Scatchard Analysis of Prolactin Binding.

Milk microsomes treated with 5M $MgCl_2$ were prepared from preterm (pooled) and fullterm (pooled) milk collected sixteen days after delivery between 6-7 am. Values are means of duplicate determination. The binding capacity and dissociation constant of Prolactin binding were calculated from linear regression analysis ($n = 9$ $r = -.625$; $n = 8$ $r = -.603$ ($p < .05$)). Preterm ⊖—⊖—⊖ Fullterm ⊖·⊖·⊖

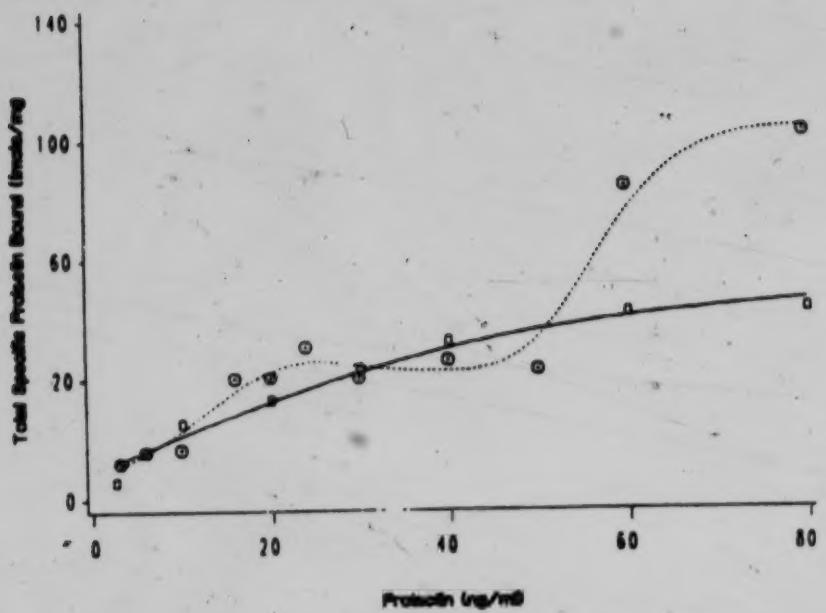

Figure III-6. Relationship between total bound prolactin and prolactin concentration.

Milk microsomes treated with 5M $MgCl_2$ were prepared from preterm (pooled) and fullterm (pooled) milk collected sixteen days after delivery between 6-7 am. Total prolactin bound is expressed as the total specific bound prolactin per mg of membrane protein. The specific bound prolactin was 45-65% of total prolactin bound. Values at each prolactin concentration are means of duplicate determination. Preterm ●—●—● Fullterm ⊙ ⊙-⊙

over the prolactin concentration range of 0-80 ng/mL. Scatchard analysis (Scatchard, 1949) of binding of ^{125}I-human prolactin to milk microsomal membrane showed one class of high capacity (116.9 $\pm$ 12.4 fmol/mg protein), low affinity (4.2$\pm$ 0.4 x 10^{-9}M) binding sites.

Effect of Stage of Gestation and Duration of Lactation on Prolactin Receptor Content of Milk

Total receptor content of milk significantly ($p<0.05$) increased over the duration of lactation in both groups (Figure III-7). Mean receptor content (mean $\pm$ SD) on days 4, 16 and 37 postpartum for preterm and fullterm samples were 66.2 $\pm$ 28.2, 138.5 $\pm$ 28.2, 126.7 $\pm$ 28.2 (fmol/mg protein) and 72.8 $\pm$ 21.3, 103.1 $\pm$ 28.2, 161.1 $\pm$ 28.2 (fmol/mg protein), respectively. No effect of gestational age on the binding capacity of the receptor was observed over the course of the study or between collection times. However, there was a significant difference ($p<0.05$) in receptor content (fmol/mg protein) between times of collection on the same collection day. For example, the mean receptor content of preterm milk collected between 8-9 am (T2) was significantly higher than milk expressed between 6-7 am (T1) on day 16. When receptor content was expressed per mL of milk or corrected for variations in milk volume (Figure III-7), the same profile of change in receptor content was observed.

Comparison of prolactin binding at each prolactin concentration used with the stage of gestation, duration of lactation and time of collection was done to assess whether gestational effects could be detected (data not

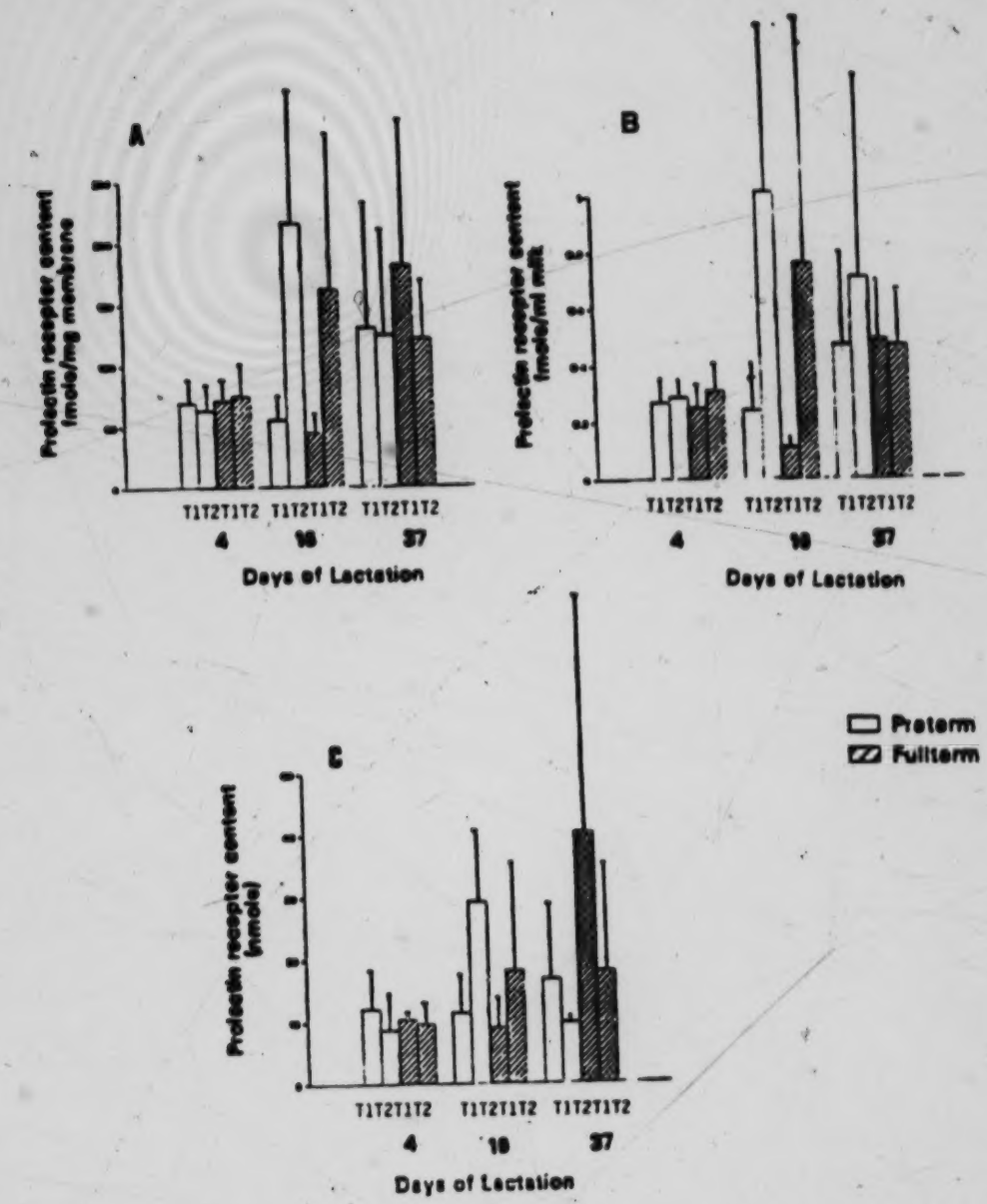

Figure III-7. Effects of gestation and lactation on prolactin content in human milk.

Milk microsomes treated with 5M $MgCl_2$ were prepared from from milk collected from mothers delivering prematurely (28 - 34 weeks) and at term. Milk samples were collected 4, 16 and 37 days postpartum between 6-7 am (T1) and 8-9 am (T2). Milk from two subjects was pooled for the same day and time on days 4, 16 and 37 for both groups (n = 5; pooled). Two sets of four subjects were pooled for the fullterm group on day 4 (n = 3; pooled). A. Prolactin receptor content in milk (fmole/mg) membrane protein. B. Prolactin receptor content in milk (fmole/ml milk). C. Total prolactin receptor in milk (nmoles).

illustrated). However, differences in prolactin binding at each level of prolactin studied could only be attributed to changes occurring with maturation of lactation. These results suggest that differences in the rates of appearance of prolactin receptor in milk exist between groups. This observation is not statistically significant however, as marked within group variations in prolactin binding was observed.

Effect of Stage of Gestation and Duration of Lactation on the Binding Affinity of the Prolactin Receptor

No significant difference in the affinity constant (K_a) of the milk receptor was detected between groups and collection times over the course of the study (data not illustrated).

Effect of Stage of Gestation and Duration of Lactation on the Concentration of Prolactin in Milk

The validity of milk prolactin measurement was assessed by examining the immunoreactivity of milk prolactin with serum standards (Figure III-8). The interassay and intraassay coefficient of variation of this assay was 17.4 $\pm$ 2.1 and 2.5 $\pm$ 1.1, respectively (Figure A-IV).

The concentration of prolactin decreased significantly ($p<0.05$) in both groups over the three collection days in a manner reflecting the role of prolactin in the early stages of lactation. Mean ($\pm$ SD) concentration of prolactin in milk on 4, 16 and 37 days postpartum were 25.3 $\pm$ 11.7, 16.4 $\pm$ 12.1, and 13.7 $\pm$ 4.4 ng/mL, respectively (Figure III-9). No effect of gestational age or of time of collection on the concentration of prolactin in

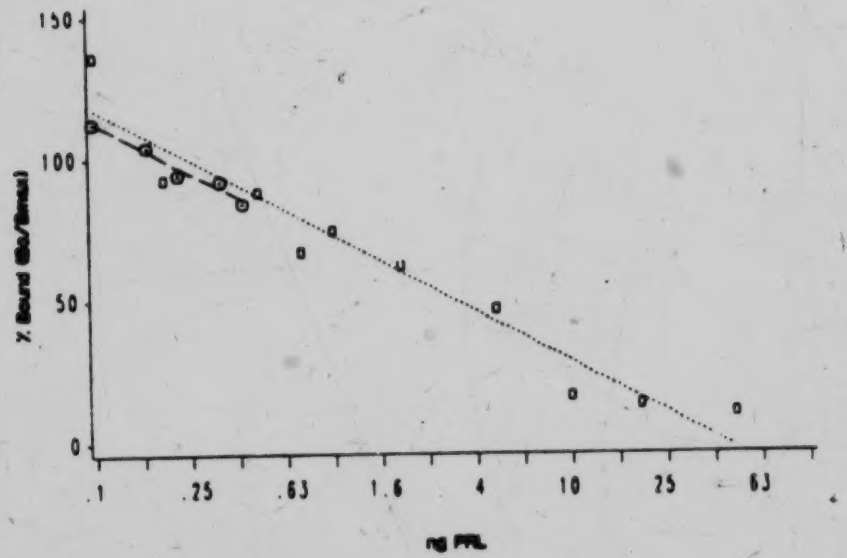

Fig III-8. Standard Curve of Prolactin Determination.

Plot of decrease in the percentage of ^{125}hPRL bound to antiserum (log scale) with increasing amounts of standard human prolactin (ng) o o o or diluted human milk (ug) o–o–o all compared in a single radioimmunoassay. Regression coefficients were significant ($r = -.968$, $n = 10$; $p < .06$).

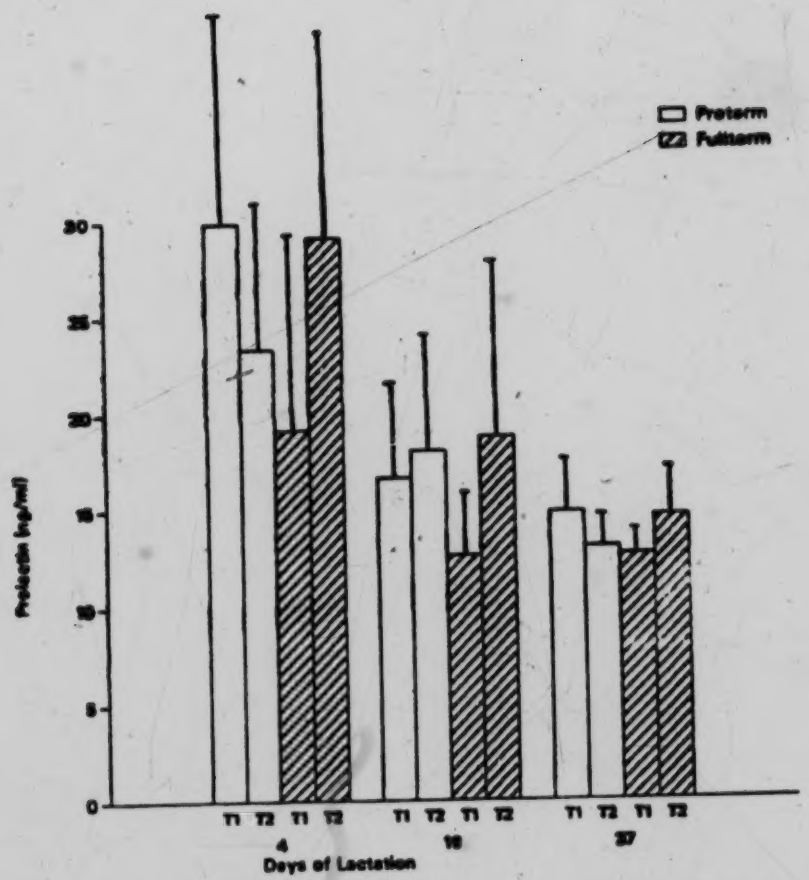

Figure III-9. Effects of gestation and lactation on prolactin concentration in human milk.

Prolactin was measured in defatted milk samples diluted 1:2.5 in phosphate buffered saline/1% BSA pH 7.45. Milk was collected 4, 16 and 37 days postpartum between 6-7 am (T1) and 8-9 am (T2). Milk from two subjects was pooled on each day and time (n = 5). Milk from fullterm subjects on day 4 was a pool of two sets of pools for each time (n = 3). The decrease in prolactin concentration over duration of lactation was significant ($p < .05$).

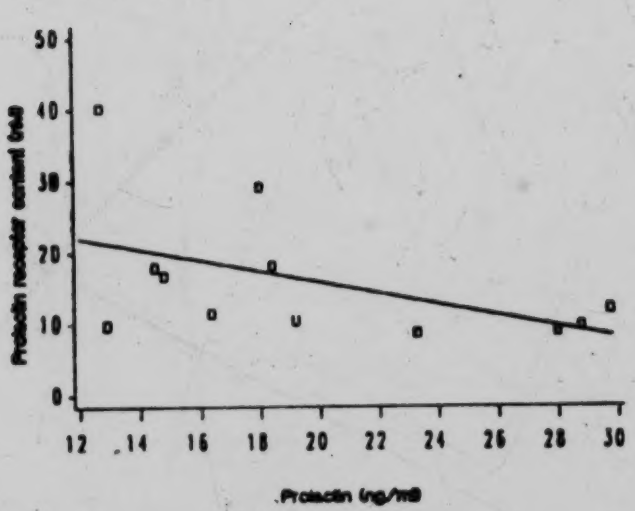

Figure III-10. Relationship between prolactin concentration and prolactin receptor content in human milk.

Correlation between milk prolactin concentration and prolactin receptor levels was determined in samples collected between 6-7 am (T1) and 8-9 am (T2) on days 4, 16 and 37 postpartum as described previously. Total prolactin receptor levels were calculated by multiplying the binding capacity (fmole/mg membrane) by the total amount of milk microsomal protein (treated with 5M $MgCl_2$) in each expression. Values are means (n=5) for all days in both groups (n=3, Day 4 Fullterm). r = -.498 n = 56 ($p < .05$).

milk was observed. The levels of milk prolactin showed no significant correlation with milk volume.

Relationship Between the Prolactin Receptor Content and the Concentration of Prolactin in Milk

The concentration of prolactin in milk was compared with the prolactin receptor content of preterm and fullterm milk to assess whether a relationship between these factors exist. The concentration of prolactin in preterm and fullterm milk was negatively correlated with the prolactin receptor content in milk ($p<0.05$) (Figure III-10) in both groups over the course of the study.

Effect of Stage of Gestation and Duration of Lactation on the Nutrient Composition of Milk

The concentration of protein in preterm milk was significantly ($p<0.05$) higher than fullterm milk over the duration of lactation (Figure III-11). The mean ($\pm$ SD) concentration of protein in preterm and fullterm milk 4, 16 and 37 days postpartum was 22.6 $\pm$ 10.5, 20.2 $\pm$ 4.3, 17.9 $\pm$ 5.9 mg/mL and 15.4 $\pm$ 12.5, 20.2 $\pm$ 7.9, 17.9 $\pm$ 12.9 mg/mL, respectively. No apparent effect of time of collection or the stage of lactation on the concentration of protein in the milk over the course of the study was observed. However, when the protein levels in milk were corrected for differences in milk volume, a significant ($p<0.05$) effect of time of expression was observed (data not illustrated). The content of protein in the first early morning expression (T1) was significantly higher ($p<0.05$)

than those in the milk collected two hours later (T2). These results reflect difference in milk volume produced in each expression (Table III-2).

No apparent effect of gestational age, duration of lactation and time of collection on the concentration of fat was observed in this study (Figure III-12). Mean (± SD) concentration of fat in preterm and fullterm milk was 2.3 ± 1.1, 2.3 ± 0.8, 2.9 ± 1.7 g/dl and 1.6 ± 1.3, 3.2 ± 3.2, 2.1 ± 0.9 g/dl, respectively. Research has shown that heating milk samples to 37°C before aliquoting milk samples is necessary in order to allow complete mixing of milk constituents (Ferris & Jensen, 1984). This was not done in this study which may account for the large within group variability in fat determination that was observed. Pooling of milk may have also masked the effect of gestational age, duration of lactation and time of collection on the concentration of fat. Correction for volume difference in each expression did not alter the pattern of results (data not illustrated).

The concentration of lactose in preterm milk was significantly higher $p<0.05$) than fullterm milk over the duration of the study (Figure III-13). Mean (± SD) lactose concentration of preterm and fullterm milk 4, 16 and 37 days postpartum were 101.2 ± 31.0, 123.9 ± 57.3, 111.9 ± 40.5 mM and 83.6 ± 57.6, 85.3 ± 50.7, 91.3 ± 24.3 mM, respectively. No apparent effect of duration of lactation and time of collection on the concentration of lactose in milk was observed. However, when values were corrected for variations in volume in each expression (data not illustrated), a significant difference in lactose content was observed between time of collection. The lactose content of milk in the first early morning (T1

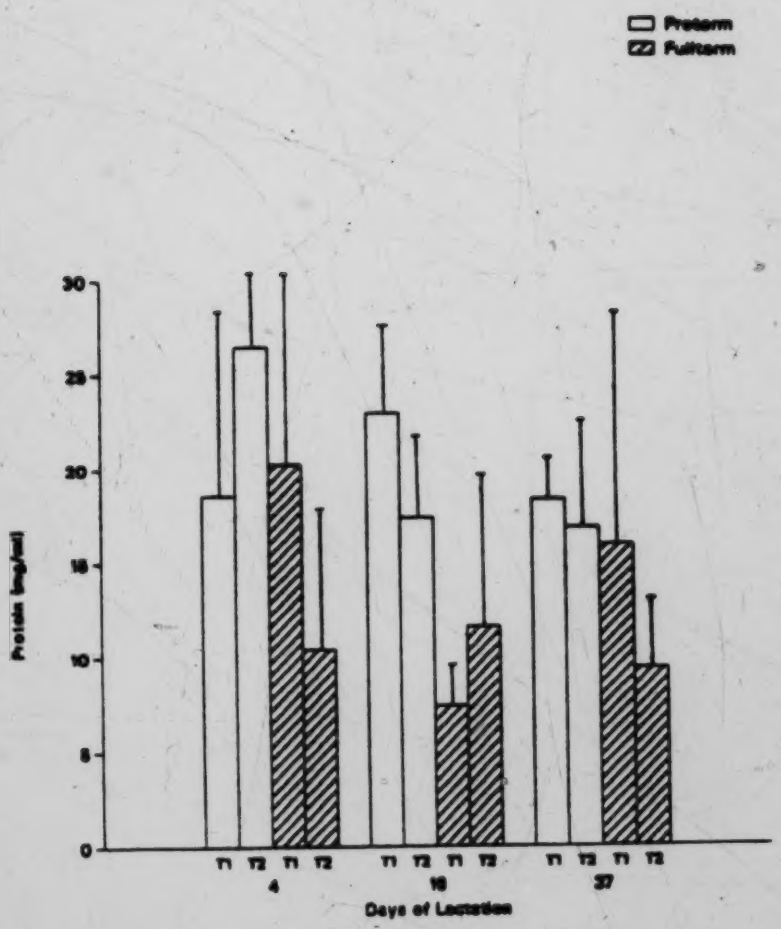

Figure III-11 Effects of gestation and lactation on protein concentration in human milk.

Protein was measured in defatted milk samples diluted 1:45 in 25 mM TRIS-HCL, 10 Mm $MgCl_2$ pH 7.5. Milk was collected 4, 16 and 37 days postpartum between 6-7 am (T1) and 8-9 am (T2). Milk from two subjects was pooled on each day and time (n = 5) for both groups with the following exception: fullterm milk samples collected on day 4 was a pool of four subjects for both times (n = 3). The protein concentration of the preterm milk was significantly higher ($p < 0.5$) than the fullterm milk over duration of lactation.

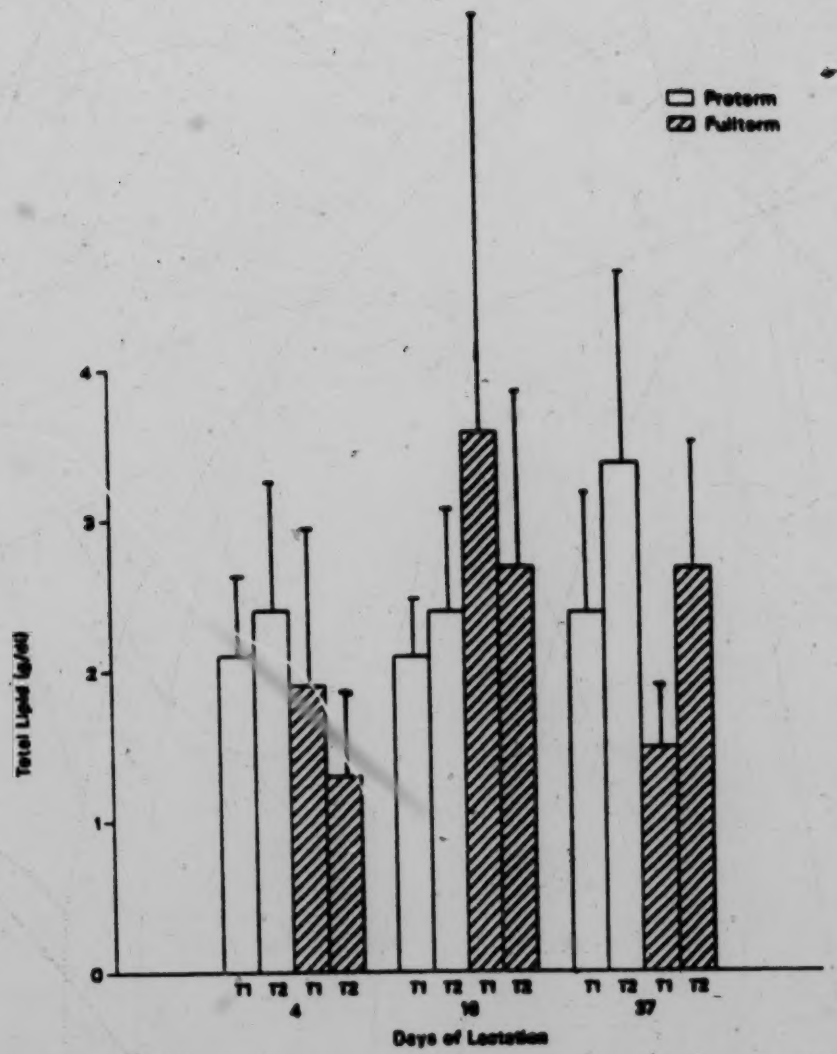

Figure III-12. Effects of gestation and lactation on lipid concentration in human milk.

Fat was extracted from 1.5 ml milk samples using a modified Folch procedure as described previously. Milk was collected 4, 16 and 37 days postpartum between 6-7 am (T1) and 8-9 am (T2). Milk from two subjects was pooled on each day and time (n = 5) for both groups. Fullterm milk collected four days postpartum (T1 and T2) represent a pool of four subjects (n = 3).

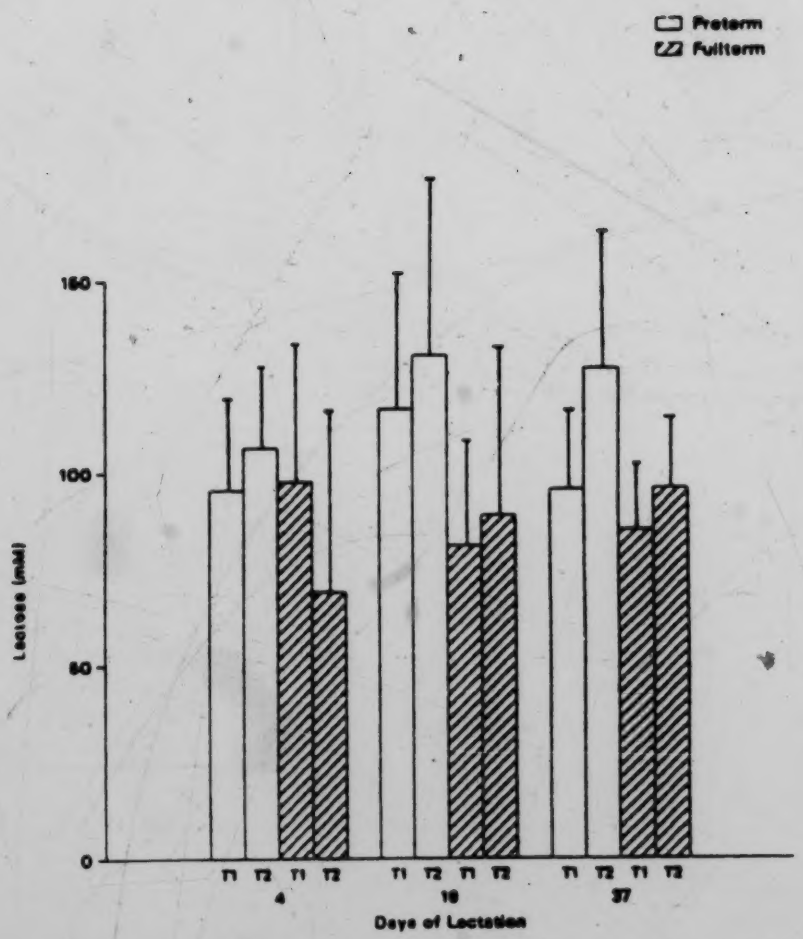

Figure III-13. Effects of gestation and lactation on lactose concentration in human milk.

Lactose was measured in milk samples diluted 1:50 in dionized water as described previously. Milk was collected 4, 16 and 37 days days postpartum between 6-7 am (T1) and 8-9 am (T2). Milk from two subjects was pooled on each day and time (n = 5) for both groups. Fullterm milk collected four days postpartum (T1 and T2) represent a pool of four subjects (n = 3). The concentration of lactose in preterm milk was significantly higher ($p < .05$) than fullterm milk over duration of lactation.

was significantly ($p<0.05$) higher than the second collection (data not illustrated).

Relationship Between the Prolactin Receptor Content, the Concentration of Prolactin and the Nutrient Composition of Milk

There was a strong negative correlation ($r=-0.699$) between the concentration of prolactin and the total fat content in preterm and fullterm milk over the course of the study (Figure III-14). There was also a significant ($p<0.05$) negative correlation ($r=-0.553;n=12$) between milk prolactin and lactose values (Figure III-15) over the course of the study. These relationships may demonstrate the declining role of prolactin on the nutrient composition of milk as lactation proceeds.

There was a weak positive correlation between milk prolactin receptor content and the protein and lactose content (data not illustrated). However, these relationships were not significant over the course of the study. Large within group variability of receptor content (Figure III-7), lactose and protein content (Figure III-11, 13) on Day 16 and 37 indicate that a range of individual response may be occurring. For example, on Day 16, the large within group variability in receptor content was associated with a corresponding high level of variability in the nutrient composition of milk. These results suggest that a range of prolactin receptor content may exist in each pool of milk, resulting in a large variability in the nutrient composition. However, further research is required to asses the effect of within group variability on determinations of nutrient composition of milk.

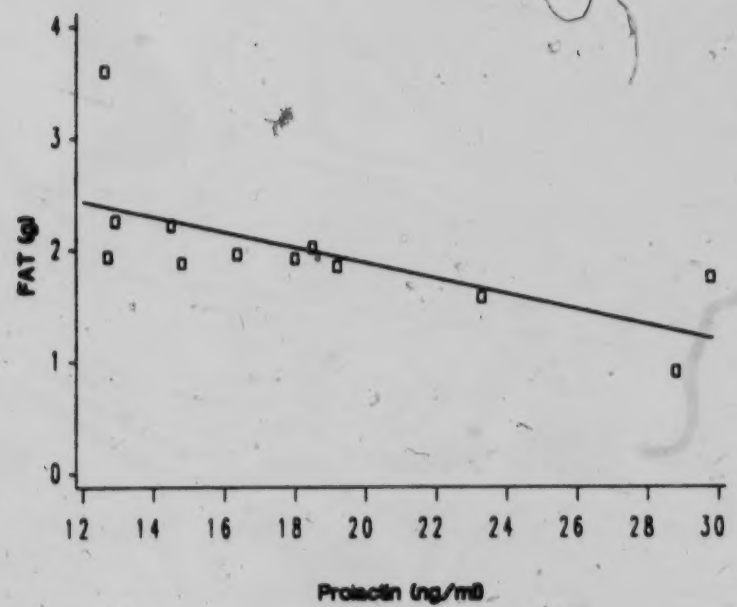

Figure III-14. Relationship between prolactin concentration and total fat in milk.

Correlation between milk prolactin concentration and fat content was determined in samples collected between 6-7 am (T1) and 8-9 am (T2) on days 4, 16 and 37 postpartum. Values are means ($n = 5$) for all days and times in both groups except on day 4 ($n = 3$ fullterm (T1 and T2)). $r = -.699$ $n = 12$ ($p < .05$).

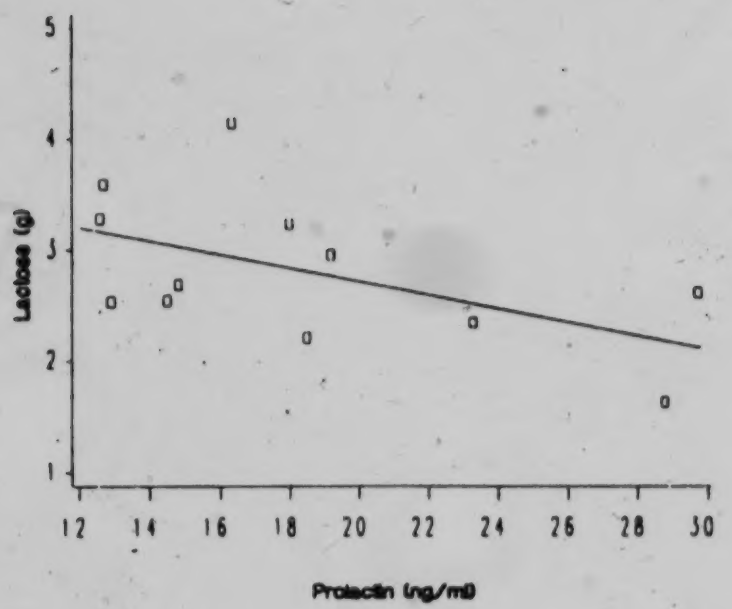

Figure III-15. Relationship between prolactin concentration and total lactose in milk.

Correlation between milk prolactin concentration and lactose content was determined in samples collected between 6-7 am (T1) and 8-9 am (T2) on days 4, 16 and 37 postpartum. Values are means ($n = 5$) for all days and times in both groups except on day 4 ($n = 3$ fullterm (T1 and T2)). $r = .553$ $n = 12$ ($p < .05$).

D. DISCUSSION

Properties of Prolactin Binding in Milk

The present study demonstrated that a prolactin binding protein that selectively binds prolactin exists in human milk. Milk prolactin receptors were isolated from crude microsomal fractions in agreement with sites previously reported in rabbit milk (Waters et al., 1980) and mammary gland (Waters et al., 1984; Bohnet et al., 1976; Djiane et al., 1977; Katoh et al., 1984; Posner et al., 1974; Shiu & Friesen, 1974). Molecular mass estimations for this binding protein were significantly higher than those reported for highly purfied membranes (Dusanter-Fourt et al., 1987; Waters et al, 1984; Sakai & Ike, 1986). Recently, two binding subunits with molecular masses of 36,800 and 83,200 (high K_d) daltons were isolated in rabbit mammary gland (Sakai & Ike, 1987). This suggests that the receptor purified in this study may represent an aggregate of these two binding proteins as the total weight of the two binding subunits are not significantly different from the molecular size estimation in this study. However, further research is required to establish the effects of receptor purification on the properties of prolactin receptor binding in human milk.

Binding of prolactin increased with treatment of microsomal membrane with 5M $MgCl_2$. This procedure resulted in the removal of endogenously bound prolactin from the binding sites (Kelly et al., 1979). Conditions for maximal specfic binding were similar to those reported in the literature (Waters et al., 1980; Djiane et al., 1977; Shiu & Friesen, 1974). Binding increased with membrane protein concentration and length

of incubation. Binding of prolactin to its receptor was saturable, slowly reversible ($t_{1/2}$= 8-10 hours), and specific for human prolactin, indicating that Scatchard analysis was appropriate for the determination of binding capacity and affinity. This analysis revealed the existence of a high capacity/low affinity prolactin binding site in milk microsomal membrane in contrast to the higher affinity sites previously reported in rabbit milk (Waters et al., 1980) and mammary gland (Waters et al., 1984; Kelly et al., 1984; Djiane et al., 1977). Binding specificity data obtained with the milk prolactin receptor preparation was similar to that observed in other prolactin responsive tissues (Waters et al., 1980; Shiu & Friesen, 1974; Buntin & Ruzycki, 1987; Bramley et al., 1987; Kelly et al., 1984). These results indicate that this receptor is only responsive to lactogenic hormones. The fact that porcine prolactin also bound to the receptor preparation suggests that a common structural property exists in prolactin binding sites between species (Posner et al., 1974; Shiu & Friesen, 1976).

Relationship of Stage of Gestation and Duration of Lactation on Properties of Prolactin Binding

The present study demonstrated that the prolactin receptor content of milk increases with maturation of lactation. This is in agreement with patterns of receptor content observed in rabbit milk over the first four weeks of lactation (Waters et al., 1980). Prolactin receptor levels in preterm milk acheived maximal levels by day 16 of lactation and then declined by day 37 (Figure III-7). In contrast, the receptor content of fullterm milk achieved maximal levels by day 37. No apparent effect of

gestation on receptor content was observed in this study. However, differences in receptor content between groups on day 16 and 37 were observed. This observation, however, was not statistically significant due to marked within group variations in receptor content. Differences in receptor content were also observed between times of collection on day 16. The lower levels of prolactin receptor in the first early morning expressions may reflect the rapid turnover of mammary receptors. Reports in the literature have shown that the half life of the prolactin receptor in rat liver was 40-50 minutes (Baxter, 1985). In this regard, a significant quantity of mammary receptors could have been recycled or degraded in the six to eight hours remaining since the last time of expression, thus potentially explaining the lower receptor levels in the first early morning expressions. These changes in receptor content occurred in concert with a marked decrease in the concentration of prolactin in milk after Day 4 (Figure III-9). These changes in the concentrations of prolactin reflect the declining role of prolactin as lactation progresses (Healy et al., 1980; Gupta, 1983).

Comparison of these changes in milk composition displayed a significant ($p<0.05$) negative relationship between milk receptor and prolactin levels. Waters et al. (1980) demonstrated that milk prolactin receptor content was directly correlated with mammary gland prolactin receptor content over the duration of lactation. It is possible then, that changes in prolactin receptor content that occur with duration of lactation reflect changes in mammary gland receptor content as shown by Djiane et al. (1977). In addition, milk prolactin levels may reflect changes in maternal

serum levels as reported by other investigators (Gala, 1983; Mulloy & Malven, 1979). Thus, the drop in the concentration of prolactin in milk observed in this study may reflect coinciding decreases in serum levels of prolactin. This decrease in the concentration of prolactin may trigger a signal to modulate receptor function in the mammary gland by increasing the number of available binding sites. Changes in binding affinity are unlikely as no apparent effect of gestational age, duration of lactation and time of collection was observed in this study. These findings are in agreement with those found in the literature (Waters et al., 1980; Waters et al., 1984; Kelly et al., 1984). The possibility that other hormones may cause a desaturation of previously occupied sites by the withdrawal of some endogenous placental mammotroph is unlikely as treatment of the membrane with 5M $MgCl_2$ was necessary before significant levels of specific binding could be detected in the microsomal fractions of milk. Thus, further research is required to establish the role of other hormones (in particular, progesterone and estradiol) on prolactin receptor function during lactation. Measurement of these factors in milk may provide an easier, non-invasive method to establish the role of these factors on prolactin receptor function.

It is noteworthy to point out that the profiles of prolactin receptor content in human milk over duration of lactation reported in this study parallel longitudinal patterns of 6-Keto-prostaglandin $F_{1\mu}$ and prostaglandin E content of fullterm milk (Chappell et al, 1983). These compositional patterns can not be directly interpreted. However, it seems likely that these profiles may reflect the role of prostaglandins in prolactin receptor

mediated functions. Prolactin has been shown to modulate its own receptor by increasing the fluidity of the membrane in which it exists by stimulating prostaglandin synthesis (Dave et al., 1982). These changes in membrane fluidity have been associated with an increase in the number of available binding sites (Dave & Witorsch, 1984). In this regard, the profile of prolactin receptor content in milk observed in this study may be associated with changes in membrane fluidity over the duration of lactation. A comparison between receptor numbers and prostaglandin content of fullterm milk at different stages of gestation and duration of lactation would be useful to assess the balance of hormonal subcellular controls over lactation. Chapter IV will discuss the effect of fatty acid composition of membrane phospholipids on prolactin binding properties.

Relationships of Stage of Gestation and Duration of Lactation to the Nutrient Composition of Milk

In this study, the concentration of lactose and protein in preterm milk was significantly higher than fullterm milk. No effect of stage of lactation or time of expression was observed. However, when values were corrected for variations in milk volume the protein and lactose content in milk was significantly higher in the first early morning expressions. No effect of gestational age, duration of lactation or time of expression on the concentration of fat was observed in this study. A marked within group variability may account for the failure to distinguish any significant gestational effects in the concentration of fat in milk. Reports of the nutrient composition of milk at different stages of gestation

and lactation vary among investigators (Atkinson et al., 1982; Moran et al., 1983; Ferris & Jensen, Davey et al., 1982). The method of milk collection and the handling and storage of milk specimens also affects determinations of the nutrient composition (Chappell et al., 1985). The results reported herein, are comparable to the variations in nutrient composition reported by these investigators. For example, most investigators, agree that the lipid content of preterm milk is significantly higher than fullterm milk. In this study no effect of gestation on the concentration of fat was observed. This observation however, was probably due to the handling of milk specimens by the investigator. Failure to properly mix milk samples at 37°C at the time of collection may have resulted in incomplete mixing of milk constituents and in hydrolysis of milk fats. Thus, these factors may have contributed to the marked within group variability in nutrient and prolactin binding determinations.

Relationship between the Prolactin Receptor Content, the Concentration of Prolactin and the Nutrient Composition of Milk

This study demonstrated a negative correlation between the concentration of prolactin and the lipid and lactose content of milk. This is in agreement with reports in the literature (Healy et al., 1980). In addition, prolactin receptor levels were positively correlated with the protein and lactose content in milk (data not illustrated). However, the relationship was not statistically significant as marked within group variability was observed in the receptor and the lactose content of milk (Figure III-13). This relationship was most apparent in the fullterm

samples collected between 8-9 am. These results suggest that a relationship between maternal hormonal status as reflected in prolactin receptor content and the concentration of prolactin in milk, with the nutrient composition, exists over the duration of lactation. However, as gestational effects were not observed in this study, it is likely that other factors in addition to prolactin play a role in determining milk composition. Research has indicated that progesterone and estradiol have a direct effect on prolactin receptor function in the mammary gland (Bohnet et al., 1976; Erb et al., 1977; Guillanmot etl al., 1986; Kulski et al., 1977; Sakai & Ike 1987; Sakai & Banerjee, 1979). Procedures for collecting and handling milk have also been shown to affect determinations of milk content (Chappell et al., 1985; Bjorksten et al., 1980; Ferris & Jensen, 1984; Friend et al., 1983). It is likely that handling of milk samples at the time of collection influenced the determinations of prolactin and nutrients in milk. For example, heating of milk to 37°C inactivates lipase activity (Wardell et al., 1984) and allows complete mixing of the milk constituents. This was not done at time of collection indicating that samples aliquoted and stored frozen at -70°C may have been heterogeneous. It is also possible that losses in protein and lipids occured through the adherence of these nutrients to milk containers. In addition, the small sample size used in this study, the large within group variability observed and pooling of milk may have directly masked the effect of gestation. Further research is required to establish the role of other hormones on prolactin receptor function at different stages of gestation and duration of lactation.

In conclusion, the present study demonstrated that a prolactin binding protein that selectively binds prolactin exists in human milk. Moreover, this study suggests that the underlying hormonal events affecting prolactin binding and the nutrient compositin of milk are complex in nature, and are not soley due to prolactin.

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IV. EFFECTS OF MEMBRANE COMPOSITION ON THE PROPERTIES OF PROLACTIN BINDING

A. INTRODUCTION

There is evidence in the literature to suggest that diet-induced compositional change in the membrane lipid bilayer alters hormone receptor mediated function (Clandinin et al., 1985). Although little research has been done for prolactin, there is some evidence to suggest that change in membrane lipid composition affects the function of the receptor (Dave & Witorsch, 1985). For example, prolactin binding to its membrane receptor has been associated with changes in membrane fluidity (Bhattachaya and Vonderhaar, 1981; Dave et al., 1982) in an age dependent manner (Dave & Witorsch, 1985). It is thought that prolactin may mediate these increases in membrane fluidity by stimulating prostaglandin synthesis (Dave et al., 1982) resulting in changes in the lipid microenvironment surrounding the prolactin receptor and in an increase in the number of available binding sites. Diet-induced changes in membrane composition have also been associated with changes in prolactin binding. Cave & Jurkowski (1984) demonstrated in the rat model, that changes in dietary fat resulted in alterations in specific binding of prolactin in mammary and hepatic tumours initiated by N-methyl-N-nitrosurea. These reductions in prolactin binding occurred when the dietary polyunsaturated levels fell below three percent suggesting that an unknown level of lipid desaturation must be reached before changes in membrane fluidity result in optimal levels of prolactin binding in human milk.

It has been demonstrated that human milk contains a prolactin receptor (Clandinin et al., 1986) that selectively binds human prolactin. We also demonstrated that the prolactin receptor content of human milk increased with duration of lactation (Chapter III). These changes were paralleled by changes in the concentration of prolactin and to some extent by transitions in the nutrient composition of milk over the length of lactation. The present study was designed to examine the effect of fatty acid composition of microsomal membrane phospholipids on prolactin receptor binding at different stages of gestation and duration of lactation.

B. MATERIALS AND METHODS

Pooling of Membranes

Microsomal membranes were isolated from preterm and fullterm milk collected in a manner described previously (Chapter III). Approximately 1.0 mg of microsomal membrane protein was resuspended in 100-500 µl of 25 mM TRIS-HCl, 10 mM $MgCl_2$ pH 7.5 and stored frozen at -70°C until assay.

Microsomal membranes isolated from five pooled preterm milks were pooled by day and time to 1) ensure adequate quantities of material for fatty acid analyses, 2) to assess the effect of gestational age and duration of lactation on the fatty acid composition of microsomal phospholipids and, 3) to compare the fatty acid composition of microsomal membranes with the properties of prolactin receptor binding at these different stages.

Microsomal membranes prepared from five pooled fullterm milks collected at different stages of gestation and lactation were pooled in two ways. Membrane samples were pooled for the second time of expression (T2) by increasing receptor content to allow comparisons between fatty acid composition of microsomal phospholipids to be made with increasing levels of available binding sites. Membranes from the first collection (6-7 am; T1) period were pooled by day to allow comparison between membrane fatty acid composition with prolactin receptor binding over the duration of lactation.

Prolactin binding was measured using the method described previously (Chapter III).

Lipid Extraction

Lipids were extracted by a modified Folch procedure (Folch et al., 1957). Microsomal membrane was (100-500 ul) added to 20 mL of Chloroform (C):Methanol (M) (2:1) containing 0.1% (w/v) ethoxyquin in borosilicate glass tubes (16 m x 125 m). Samples were vortexed vigorously and left to stand at 4°C overnight. At the end of this period, 4 mL of 0.9% (w/v) sodium chloride (NaCl) was added to the tubes. Tubes were mixed vigorously and the top layer removed with a Pasteur pipette. The lower phase was transferred into a clean borosilicate glass test tube and then centrifuged at 40°C under vacuum (Model RH-29 Speed Vac Concentrator Centrifuge; Savant Instruments Inc., Ont.) for three hours to evaporate the chloroform. The lipids were resuspended in hexane and

transferred to 1.8 mL vials with teflon lined caps and stored at -70°C until assay.

Separation of Lipids

Phospholipids were separated using the method of Touchstone et al. (1980). Individual phospholipids were separated on Whatman HP-K thin layer chromatography plates (10 x 10 cm) using the following solvent system: chloroform: methanol:2-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:18 by volume) (Touchstone et al., 1980). Separated phospholipids were sprayed with 0.03% w/v 2'7'dichlorofluorescein in 0.01 M NaOH and detected by comparison under ultraviolet light with appropriate standards. The bands were scraped into methylation tubes for fatty acid analysis.

Fatty Acid Analysis

Fatty acids were methylated using 14% w/v BF_3/methanol reagent (Metcalfe & Schmidtz, 1961) in the following manner. Distilled hexane (1 mL) and 1 mL of BF_3 (14% w/v) were added to methylation tubes containing the phospholipids. The tubes were tightly capped and heated in a sand bath at 100-110°C for 1 hour. After the samples cooled, 1 mL of distilled water and 0.8 mL of hexane was added to the tubes and the samples vigorously vortexed. The hexane layer was removed into clean 1.8 mL vials and the methyl esters dried at 40°C under vacuum (Model RH 12-29 Speed Vac Concentrator Centrifuge; Savant Instruments Inc., Ontario). The vials were then flushed with N_2, capped with teflon lined caps and stored at -70°C until analysis by capillary gas liquid chromatography.

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were separated by automated gas-liquid chromatography (Vista 6000 GLC and Vista 654 data system, Varian Instruments, Georgetown, Ontario) using a fused silica BP 20 capillary column (25 m x 0.25 m i.d.; Varian, Georgetown, Ontario). Helium was used as the carrier gas at a flow rate of 1.8 mL/min using a splitless injection mode. The initial oven temperature (90°C) was increased to 172°C at 20°C/min, held for 13.2 minutes, and then increased to 220°C at 3.5°C/min. The total time of analysis in this method was 45 min. This method allowed identification of all saturated, polyunsaturated and monounsaturated fatty acids.

Statistical Analysis

The effect of stage of gestation and duration of lactation on the fatty acid compostion of microsomal phospholipids was compared by Least-squared anlysis of variance procedures (Harvey, 1975). Regression analysis was used to compare the fatty acid composition of microsomal phospholipids with prolactin binding (Steele & Torrie, 1980).

C. RESULTS

Effect of Stage of Gestation and Duration of Lactation on the Fatty Acid Composition of Membrane Phospholipids

Phosphatidylcholine

Major fatty acids in phosphatidylcholine were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 32.8%, 23.4%, 19.3% and 18.6% (w/w), respectively, in

microsomes isolated from full term milk. Major fatty acids in preterm milk microsomes were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 44.4%, 26.4%, 12.2% and 9.1% (w/w) respectively. Fullterm microsomes exhibited significantly ($p<.05$) higher percentages of $C_{18:2(6)}$ and $C_{18:1(9)}$, compared to microsomes isolated from preterm milk over the duration of the study (Table IV-1). In addition, microsomal membranes isolated from fullterm milk were characterized by significantly higher levels of monounsaturated, polyunsaturated and w6 fatty acids when compared to microsomes isolated from preterm milk (Table IV-1;$p<.05$). In contrast, milk microsomes isolated from preterm milk had significantly higher levels of $C_{16:0}$ and saturated fatty acids ($p<.05$). A significant difference in $C_{18:2(6)}$, $C_{16:0}$, w6, and polyunsaturated fatty acid content was observed between the times of collection in both groups over the duration of lactation. No effect of duration of lactation on the fatty acid composition of phosphatidylcholine was observed in this study.

Phosphatidylethanolamine

Major fatty acids in phosphatidylethanolamine were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 14.9%, 31.8%, 25.6% and 18.6%(w/w), repectively, in microsomes isolated from fullterm milk. Major fatty acids of phosphatidylethanolamine present in microsomal membranes isolated from preterm milk were $C_{16:0,\ C18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 14.8%, 28.9%, 25.6% and 19.2%(w/w), respectively (Table IV-1). Microsomes isolated from preterm milk had significantly higher content of $C_{18:0}$, $C_{18:1(9)}$ and monounsaturated fatty acids when compared to fullterm microsomes over

the course of the study ($p<.05$). Microsomes isolated from fullterm milk were characterized by significantly higher levels of saturated fatty acids ($p<.05$). This relationship was most apparent in the membranes isolated from milk collected between 8-9 am. No effect of duration of lactation was observed on the fatty acid composition of phosphatidylethanolamine.

Phosphatidylinositol

The major fatty acids in phosphatidylinositol were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 12.9%, 34.2%, 22.6% and 13.9%(w/w), respectively, in microsomes isolated from fullterm milk. Major fatty acids in phosphatidylinositol were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 21.6%, 35.3%, 20.4% and 5.1%(w/w), respectively, in microsomes prepared from preterm milk (Table IV-1). Fullterm microsomes had significantly higher levels of $C_{18:1(9)}$, $C_{18:2(6)}$, w6, and polyunsaturated fatty acids compared to microsomes isolated from preterm milk ($p<.05$). Preterm microsomes were characterized by higher levels of $C_{16:0}$ and saturated fatty acids.

Phosphatidylserine

The major fatty acids in phosphatidylserine in microsomes isolated from preterm milk were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 15.2%, 30.3%, 23.4% and 19.3% (w/w), respectively. Major fatty acids in phosphatidylserine in microsomes isolated from preterm milk were $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$ and $C_{18:2(6)}$ representing 16.1%, 44.3%, 14.9% and 8.45% (w/w), respectively. Microsomes isolated from fullterm milk had significantly higher content of $C_{18:0}$, and saturated fatty acids compared to microsomes

Table IV-1. The fatty acid composition of microsomal membrane phospholipids at different stages of gestation.

Phospholipid	Time	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2(6)}$	$C_{20:4(6)}$	Σsat	Σmono-unsat	Σpoly-unsat	(Σω6)	(Σω3)
					w/w							
Phosphatidylcholine												
Fullterm	T1	39.0±.9	1.3±.8	20.5±1.8	18.8±.8^{a}	16.2±2.4^{a}	.8±.1	60.8±2.8^{a}	20.8±.3^{a}	17.8±2.4^{ac}	17.9±2.5^{ac}	.6±.3^{a}
	T2	26.7±0	.6±0	26.2±0	19.8±0^{a}	20.9±0^{b}	1.3±0	53.9±0^{b}	21.6±0^{b}	23.3±0^{b}	23.3±0^{b}	1.2±0^{b}
Preterm	T1	47.5±7.2	.5±.1	26.8±3.7	9.1±3.1^{b}	7.2±4.3^{c}	.9±.7	77.3±9.9^{c}	11.2±2.9^{c}	9.3±5.7^{c}	9.4±5.8^{ac}	2.7±3.7^{ab}
	T2	41.3±2.3	.6±.3	26.8±8.5	15.3±4.6^{ab}	11.1±2.8^{c}	1.1±.3	69.3±6.6^{ac}	17.8±4.6^{abc}	12.8±2.2^{ac}	12.9±1.6^{c}	1.3±.1^{b}
Phosphatidylethanolamine												
Fullterm	T1	12.5±2.8	1.1±0	27.7±3.9^{a}	3.7±.8^{a}	18.6±5.3	3.7±.8	42.8±6.7^{a}	32.8±3.3^{a}	26.1±4.7^{a}	24.2±5.3^{a}	1.8±.7
	T2	17.2±0	.6±0	35.9±0^{b}	2.4±0^{b}	14.2±0	2.4±0	55.5±0^{b}	25.4±0^{b}	17.7±0^{b}	17.8±0^{b}	1.4±0
Preterm	T1	15.1±.9	1.4±.4	22.5±.8^{c}	3.9±.3^{a}	18.9±.9	4.8±.3	43.3±1.5^{a}	30.6±1.7^{a}	25.8±.2^{a}	23.9±.8^{a}	1.5±.2
	T2	14.5±2.9	1.5±.6	26.3±5.5^{ac}	3.8±2.6^{ab}	19.3±4.4	3.8±2.1	44.1±6.6^{a}	34.8±4.1^{a}	21.2 ±11.4^{ab}	19.8±10.3^{ab}	1.9±.7
Phosphatidylinositol												
Fullterm	T1	10.7±9.5^{a}	1.1±.9	36.6±9.6	24.4±2.4^{a}	13.1±4.8^{a}	3.7±1.6^{ab}	49.2±1.7^{a}	27.6±4.3	19.8±6.6^{ab}	19.8±6.6^{ab}	3.3±.6
	T2	14.9±0^{ab}	1.3±0	32.2±0	20.7±0^{b}	14.7±0^{a}	3.7±0^{a}	49.4±0^{a}	26.1±0	22.8±0^{a}	22.8±0^{a}	2.5±0
Preterm	T1	21.4±5.2^{ab}	2.5±2.2	32.9±3.6	21.5±6.1^{ab}	3.6±2.8^{b}	1.7±.5^{ab}	58.7±6.8^{b}	27.6±6.1	8.3±5.1^{ab}	8.3±5.1^{b}	5.4±1.6
	T2	21.7±5.1^{a}	1.8±1.4	37.6 ±16.4	19.3±9.7^{ab}	6.5±4.9^{ab}	2.4±2.2^{ab}	62.9 ±19.4^{b}	23.6 ±11.7	10.9±8.6^{ab}	10.9±8.6^{ab}	2.5±1.6
Phosphatidylserine												
Fullterm	T1	14.8±2.0	1.1±.3	49.8±9.1^{a}	15.7±1.4^{ab}	7.5±4.2^{a}	1.3±.8^{a}	66.6±8.7^{a}	22.8±5.3	10.8±5.6^{a}	10.82±5.6^{a}	1.4±.8
	T2	17.4±0	1.1±0	38.8±0^{b}	14.1±0^{a}	9.4±0.9	1.1±0^{a}	59.8±0^{a}	24.2±0	16.9±0^{b}	13.6±0^{a}	1.3±0
Preterm	T1	17.6±5.3	.8±.2	28.2±6.9^{c}	24.7±8.4^{ab}	19.3±1.5^{b}	2.3±.4^{b}	47.9±9.6^{b}	26.7±9.1	23.5±1.1^{c}	23.5±1.1^{b}	1.9±1.9
	T2	12.8±3.0	1.1±.01	32.3±2.6^{c}	22.2±2.9^{b}	19.2±3.4^{b}	3.2±.7^{b}	46.5±.6^{b}	25.1±1.9	26.5±1.1^{d}	25.7±2.21^{b}	2.7±.4

Values are mean±SE (n=3) for fullterm and preterm (28-34 weeks) milk collected between 6-7 am (T1) and 8-9 am (T2) 4, 16 and 37 days postpartum. Values without a common superscript are significantly different ($P<.05$).

isolated from preterm milk over the duration of the study ($p<.05$). Microsomes isolated from preterm milk were characterized by higher levels of $C_{18:2(6)}$, $C_{18:1(9)}$, w6 and total polyunsaturated fatty acids. The level of polyunsaturated and w6 fatty acids was significantly higher in the microsomes prepared from milk collected between 6-7 am in both groups ($p<.05$). No effect of duration of lactation on the fatty acid composition of microsomal membranes was observed in this study (Table IV-1).

Effect of Fatty Acid Composition of Membrane Phospholipids on the Properties of Prolactin Binding

Phosphatidylcholine

No relationship between the fatty acid composition of phosphatidylcholine on the number of available prolactin binding sites in milk microsomal membrane was observed between groups or over the duration of lactation. However, when the receptor content of microsomes isolated from fullterm milk (collected between 8-9 am) was compared with the total level of polyunsaturated fatty acids in phophatidylcholine, a significant positive correlation ($r=.901$;d.f=3;$p<.05$)) was observed. Microsomes in this group were pooled by increasing receptor content (data not illustrated).

Phosphatidylethanolamine

No relationship between the fatty acid composition of phosphatidylethanolamine on the number of available binding sites in milk microsomal membrane was observed between groups or over the duration of lactation. However, when comparisons were made between receptor

content of microsomes isolated from fullterm milk (collected between 8-9 am) and the fatty acid composition, the following observations were made. Receptor levels were positively correlated with the levels of $C_{18:1(9)}$, $C_{18:2(6)}$, monounsaturated, w6 and polyunsaturated fatty acids ($p<.05$). A significant negative correlation was also observed between $C_{18:1(9)}$ and receptor content of preterm microsomes pooled by day and time (data not illustrated;$p<.05$).

Phosphatidylinositol

Linoleic acid ($r=.543;n=13$), Arachidonic acid ($r=.619;n=13$), total w6 ($r=.602;n=13$), polyunsaturated ($r=.602;n=13$) and the polyunsaturated/saturated (P/S) ratio were positively correlated with the number of available prolactin binding sites over the course of the study ($p<.05$). A significant negative correlation was observed between $C_{16:0}$ and the number of available binding sites ($r=-.581;n=13;p<.05$;data not illustrated). Significant positive correlations between $C_{18:0}$, w6 and polyunsaturated fatty acids (w/w) and the total number of binding sites in fullterm microsomes (pooled by time of collection;8-9 am) were observed (data not illustrated). Arachidonic acid content and the polyunsaturated/saturated ratio of milk microsomes pooled by day, were positively correlated ($p<.05$) with the number of binding sites (data not illustrated).

Phosphatidylserine

No relationships between the fatty acid composition of phosphatidylserine and the number of available prolactin binding sites in milk microsomal membrane was observed between groups or over the duration of lactation. However, negative correlations between $C_{16:0}$ and w3 fatty

acids (w/w) with the total number of available prolactin binding sites in fullterm microsomes collected between 8-9 am were observed (data not illustrated).

D. Discussion

Relationship between the Stage of Gestation and Duration of Lactation on the Fatty Acid Composition of Membrane Phospholipids

This study demonstrated that milk microsomes contain significant quantities of $C_{16:0}$, $C_{18:0}$, $C_{18:1(9)}$, $C_{18:2(6)}$ and smaller quantities of the longer chain polyunsaturated fatty acids. For example, the arachidonic acid content of the milk microsomes ranged between 1-4% (w/w) in contrast to liver microsomes which may vary from 10-25% (w/w) depending upon the type and level of fat in the diet (Garg et al., 1987). Individual phospholipid fractions displayed different patterns of fatty acid content between groups, time of milk collection, and over the length of lactation. No apparent relationship was observed between fatty acid content of membrane phospholipids over the length of lactation. However, significant relationships were observed between the fatty acid composition of individual phospholipid classes and the stage of gestation. For example, fullterm microsomes were characterized by higher levels of $C_{18:2(6)}$, $C_{18:1(9)}$, w6 and polyunsaturated (w/w) in phosphatidylcholine and phosphatidylinositol fractions. In phosphatidylethanolamine and phosphatidylserine fractions, preterm microsomes were characterized by higher levels of $C_{18:0}$, $C_{18:1(9)}$, monoun-

saturated fatty acids and $C_{18:1}$, $C_{18:2(6)}$, w6, polyunsaturated (w/w) fatty acids, respectively. Difference in the fatty acid content of microsomal phospholipids were also observed between times of collection. In general, the total level of polyunsaturated fatty acids differed between times of collection in the fullterm group. Although difference in fatty acid composition of microsomal phospholipids could be attributed to gestational age, the small sample size used in this study make it difficult to assess the significance of these findings. It may well be that pooling of fullterm milk microsomes (collected between 8-9 am) by increasing receptor content may have made differences between fatty acid composition of membrane phospholipids at different stages of gestation more visible. For example, the range and number of available binding sites in the pooled fullterm group (T2) was quite large when compared to the fullterm and preterm samples pooled by day and time. Thus comparison of the fullterm microsomes collected between 8-9 am with the milk microsomes pooled by day and time may not be valid as the number of samples in each pool in this group was lower and the number of binding sites significantly higher. Therefore, the method of pooling of milk microsomes may account for the gestational effects observed in this study.

Relationship Between Fatty Acid Composition of Membrane Phospholipids and Prolactin Binding Properties

The present study demonstrates that a polyunsaturated microenvironment in milk microsomes is associated with a increase in the number of available prolactin binding sites. These changes in membrane composition

do not appear to be related to the binding affinity of the receptor, as the binding affinity remained constant at different stages of gestation and lactation (ChapterIII). These findings are in agreement with those found in the literature (Dave & Witorsch, 1985; Cave & Jurkowski, 1984). Increases in prolactin binding have been associated with changes in membrane fluidity (Bhattachaya & Vonderhaar, 1981; Dave et al., 1985) and in the level of membrane content of unsaturated fatty acids (Cave & Jurkowski, 1984). Increasing polyunsaturated microenvironment is associated with localized changes in membrane fluidity which may enhance receptor mobilitiy and result in the modification of biochemical events on the cell surface (Dave et al., 1985). It has been shown that prolactin may induce localized changes in membrane fluidity through the stimulation of prostaglandin synthesis. However, evidence regarding the direct effects of changes in membrane fluidity on the action of prolactin at the cell surface remains absent in the literature.

The significance of these findings is not clear. Research has indicated that diet induced changes in membrane phospholipid composition alters hormonal mediated functions (Venkatramen et al., 1986; Clandinin et al., 1985) by altering the number of binding sites. In this study a relationship between the number of binding sites with increasing levels of polyunsaturated fatty acids in membrane phospholipids was observed at different stages of gestation and time of collection. This observation suggests that the hormonal controls of prolactin receptor function may vary at different stages of gestation. However, pooling of microsomes isolated from fullterm milk collected between 8-9 am by increasing receptor number may

have magnified the effect of gestational age on this relationship. Furthermore, the small sample size in this study makes it difficult to establish the effect of membrane composition on prolactin binding. These results have important implications for events occuring at the cell surface.

Microsomes have been shown to be responsible for the **de novo** synthesis of lipids and the recycling of receptor proteins. In this regard, the changes observed in the fatty acid composition of microsomal phospholipids and in receptor content at different stages of gestation and lactation may reflect changes that occur in the plasma membrane. Thus changes in the number of available prolactin binding sites associated with increases in the level of polyunsaturated fatty acids in microsomal phospholipids may result in changes in prolactin action at the cell surface. In turn, these changes may have potential implications for the partitioning of nutrients in milk. This study provides sufficient information to warrant further research in this area.

In conclusion, a polyunsaturated microenvironment in the milk microsomal membrane is associated with increases in the number of available prolactin binding sites. The significance of this relationship is not known. However, changes in the fatty acid composition of microsomal phosopholipids may influence prolactin action. Further research is required to establish the role of changes in membrane composition on the function of the prolactin receptor.

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V. CONCLUSIONS AND GENERAL DISCUSSION

A. Conclusions

Differences in the macronutrient composition of milk produced by mothers delivering prematurely and at term exist (Atkinson et al., 1978; Lemons et al., 1982). Maternal hormonal status (Chappell & Clandinin, 1984) and dietary intake (Neville et al., 1983; Moran et al., 1984) may influence the composition of milk. Measurement of prolactin and a prolactin binding protein may be utilized to assess prior maternal hormonal events leading to lactation.

It was hypothesized that human milk contains a prolactin receptor that selectively binds human prolactin. If specificity can be demonstrated for this receptor, then it can be specifically hypothesized for human milk that

1). Prolactin receptor content of the milk is related to the stage of gestation and lactation and thus to the hormonal status of the mother.

2). Prolactin receptor content is related to the prolactin content of the milk.

3). Prolactin receptor content is related to the total fat, protein, and lactose content of the milk.

4). The properties of prolactin receptor binding are related to the fatty acid composition of the microsomal membrane phospholipids at different stages of gestation and lactation.

The hypotheses have been verified as follows:

Hypothesis 1.

The prolactin receptor content of preterm and fullterm milk increased with the duration of lactation (Chapter III). No effect of gestational age or time of collection on receptor content in milk was observed. These results indicate that the prolactin receptor content of milk is related to the stage of lactation, in a manner that may reflect the role of prolactin in the early stages of lactation.

Hypothesis 2.

A negative correlation between prolactin receptor levels and the concentration of prolactin was observed. These results verify that milk prolactin and prolactin receptor content are directly related. This relationship has important implications for assessing maternal hormonal status at different stages of gestation and lactation. The presence of bioactive prolactin and a prolactin binding protein in human milk provide indirect evidence of an intracellular mechanism for prolactin action in the mammary gland.

Hypothesis 3.

This study demonstrated that a negative correlation exists between the concentration of prolactin and the lipid and lactose content of milk. In addition, positive trends between prolactin receptor levels and the protein and lactose content in milk were observed. These results suggest that a relationship between maternal hormonal status and the nutrient composition of milk may exist. However, as the relationship between receptor content and nutrient composition was not significant and no effect of gestational age could be established, it is unlikely that the nutrient composition of milk is only affected by prolactin.

Hypothesis 4.

This study demonstrated that an increase in the level of polyunsaturated fatty acids in milk microsomal membrane phospholipids was associated with an increase in the number of available prolactin binding sites at different stages of gestation and time of collection. Small sample size due to limited quantities of microsomal membrane made pooling of membranes by time and day necessary. Therefore, it is difficult to assess the significance of these findings. This study provides sufficent information to warrant further research in this area.

The relationships between prolactin binding and the nutrient composition of milk over the duration of lactation suggest that maternal hormonal status influenced milk composition. However, the large within group variability observed in this study and the absence of an effect of gestational age on milk composition suggest that other hormones may play a role

in partitioning of nutrients between mother and infant. For example, progesterone and estradiol may influence prolactin action by interacting with prolactin receptors in the mammary gland (Guillaumot et al., 1986; Bohnet et al., 1976). This thesis also demonstrated that changes in the level of polyunsaturated fatty acids in microsomal phospholipids were associated with increases in the number of available binding sites found. Thus binding of prolactin to its receptor may be influenced by 1) maternal hormonal environment at different stages of lactation and 2) the fatty acid composition of microsomal phospholipids. In turn, these changes in prolactin binding may influence prolactin action in the mammary gland resulting in alteration of the nutrient composition of milk.

B. FUTURE STUDIES

This thesis demonstrated that a prolactin binding protein that selectively binds prolactin, exits in human milk. These studies also indicate that the underlying hormonal events affecting prolactin binding and the nutrient composition of milk are not soley due to prolactin. Research has indicated that progesterone and estradiol have a direct effect on prolactin receptor function in the mammary gland (Bohnet et al., 1976; Sakai & Ike, 1987; Sakai & Banerjee, 1979; Guillaumot et al., 1986). Changes in membrane fluidity associated with prolactin binding have been shown to alter the number of available binding sites (Dave & Witorsch, 1984; Dave & Witorsch, 1983). It is not known how these changes in prolactin receptor function alter the nutrient composition of milk. With this in mind, studies examining the effects of hormonal regulation on prolactin receptor function

would provide useful information regarding the interaction of maternal hormonal status on the subcellular controls over lactation. For example, studies examining the interaction of progesterone and estradiol on prolactin receptor function in the mammary gland at different stages of gestation and duration of lactation would provide useful information of how changes in maternal hormonal environment during pregnancy and lactation affect prolactin receptor function. Measurement of prolactin, prolactin receptor and nutrient content of milk at these different stages would allow one to indirectly assess the effects of hormonal regulation of prolactin receptor function at the subcellular level.

Examination of the effect of diet induced changes in membrane composition on prolactin receptor function in the mammary gland would also provide useful information regarding the interaction of maternal diet on the subcellular controls of prolactin action. Analysis of milk and mammary membranes for change in prolactin binding induced by maternal diet and hormonal regulation would be useful in assessing the validity of assaying milk constituents for assessment of intracellular events. This would provide information regarding the interaction of diet and endocrine status on the subcelluar controls over lactation.

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APPENDIX I

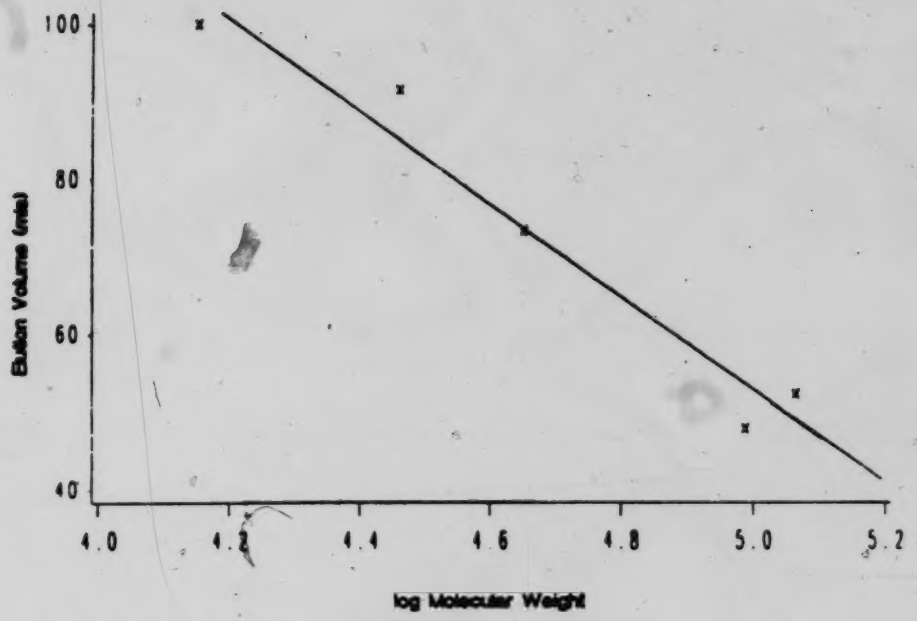

Fig AI Molecular Weight Determination of Prolactin Receptor

Estimation of molecular weight for the prolactin receptor by gel chromatography ($p < .05$). The standard protein markers used were Lactalbumin 14,200, Carbonic Anhydrase 29,000, Egg Albumin 45,000, Phosphorylase B 97,000 and Galactosidase 116,000. Blue Dextran was used to determine the void volume (50 mls).

APPENDIX II

Table A I.I Total Binding of ^{125}IhProlactin in Subcellular Fractions of Milk.

Group	20,000°g Supernatant	20,000°g pellet	100,000°g Supernatant	100,000°g pellet	2.5M $MgCl_2$ treated Microsomes	5M $MgCl_2$ treated Microsomes
			(fmole/mg)			
Preterm	2.0 ± 6[a]	11.5 ± 6.7[b]	27.9 ± 6.3[c]	18.5 ± 5.7[bc]	80.9 ± 12.8[d]	53.3 ± 13.5[d]
Fullterm	3.9 ± 2.6[a]	17.4 ± 12.9[bc]	84.9 ± 29.1[d]	62.3 ± 32.3[d]	850 ± 712[e]	611 ± 390[e]

Values are means ± SE (n = 5). Subcellular fractions were prepared from milks collected sixteen days after delivery (6-8am). Values without common superscripts are significantly different ($p < .05$).

Table A I.II Total Binding of ^{125}IhGrowth Hormone in Subcellular Fractions of Milk.

Group	20,000°g Supernatant	20,000°g pellet	100,000°g Supernatant	100,000°g pellet	2.5M $MgCl_2$ treated Microsomes	5M $MgCl_2$ treated Microsomes
			fmole/mg			
Preterm	.28 ± 07^{a}	1.2 ± .5^{b}	2.2 ± .8^{b}	1.9 ± .3^{b}	- NA -	2.7 ± .2^{b}
Fullterm	.35 ± .26^{ab}	1.1 ± .4^{b}	4.8 ± 1.6^{c}	3.8 ± 1.9^{bc}	55.4 ± 14.9^{d}	43.3 ± 15.4^{d}

Values are means ± SE (n = 5). Subcellular fractions were prepared from milks collected sixteen days after delivery (6-8am). Values without common superscripts are significantly different ($p < .05$).

Table A I.III Specific Binding of ^{125}IhPRL in Subcellular Fractions of Milk

Subcellular Fraction	Membrane Level ug	Binding Capacity fmole/mg
20,000 * g	226	1.3 ± 2[a]
pellet	452	.97 ± .1[a]
20,000 * g	680	not distinguishable
Supernatant	1360	not distinguishable
100,000 * g	331	.1 ± .09[b]
Supernatant	662	.05 ± .08[b]
5M $MgCl_2$ treated	250	5.6 ± .8[c]
Microsomal fraction	10	50.9 ± 10[d]

Values are means ± SE of duplicate determinations. Subcellular fractions were prepared from a pooled fullterm milk sample (#19) collected at 4, 16 and 37 days after delivery between 6-7 am (T1) and 8-9 am (T2). ^{125}IhPRL (25,000 cpm, .7 ng). Values without common superscripts are significantly different ($p < .05$).

Table A:II Batch differences of ^{125}IhPRL binding to milk microsomes treated with 5M $MgCL_2$.

Lot No.	[Prolactin Bound] ng/mg
410223	.103 ± .074
412064	.059 ± .034
409227	.084 ± .009

Milk microsomal membranes were prepared from a preterm milkpool (#4,5) 4 days postpartum between 6-7 am (T1). Three batches of ^{125}IhPRL (2.2 ng, 35,000 cpm) was incubated in the presence and absence of cold prolactin (2 ug/ml) with 300 ug of membrane in a final volume of .5 ml. Values are means ± SE (n = 6). Values are not significantly different ($p < .05$).

Table A III Effect of Detergent Treatment upon Prolactin Binding in Milk Microsomes

^{125}IhProlactin ng	Treatment with CHAPS	Binding Capacity fmole/mg
.8	No	.38 ± .02[a]
.8	Yes	.28 ± .23[a]
1.5	No	4.2 ± 7.5[b]
1.5	Yes	1.09 ± 5.2[b]
2.5	No	1.94 ± .44[b]
2.5	Yes	5.5 ± .65[c]

Values are means ± SE of duplicate determinations. Milk microsomes were prepared from a fullterm milk (n = 1) collected and pooled on day 4, 16 and 37 days postpartum between 6-7 am and 8-9 am. 300 ug of 5M $MgCl_2$ treated microsomes were incubated with 1m M - CHAPS for 30 minutes at room temperature with stirring. Values without common superscripts are significantly different ($p < .05$).

Table AIV Assay Characteristics of the Prolactin Radioimmunoassay

% Prolactin Recovered	Inter-assay coefficient of variation	Intraassay coefficient of variation	Blanks
109.1 ± 48	17.4 ± 2.1	2.5 ± 1.1	3.4 ± 5%

Accuracy of assay was determined by the addition of six different concentrations (1-100 ng/ml) of human PRL antigen to diluted fullterm milk samples. Interassay and Intrassay coefficients were determined using standard pools of preterm and fullterm milk collected between 4, 16 and 37 days after delivery between 6-7 am (T1) and 8-9 am (T2). Values are group means ± SE (n = 6).